SHOUT
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BIOLOGICAL BULLETIN

OF THE

Marine Biological Laboratory

WOODS HOLE, MASS.

Editorial Staff

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George T. Moore—*The Missouri Botanic Garden.*
T. H. Morgan—*Columbia University.*
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E. B. Wilson—*Columbia University.*

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Frank R. Lillie—*The University of Chicago.*

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WOODS HOLE, MASS.

JULY TO DECEMBER, 1921

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BIOLOGICAL BULLETIN

THE MARINE BIOLOGICAL LABORATORY

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Twenty-Third Year.

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I. TRUSTEES

EX OFFICIO.

Frank R. Lillie, *Director*, The University of Chicago.
Gilman A. Drew, *Assistant Director*, Marine Biological Laboratory.
D. Blakely Hoar, *Treasurer*, 161 Devonshire Street, Boston, Mass.
Gary N. Calkins, *Clerk of the Corporation*, Columbia University.

TO SERVE UNTIL 1924.

H. H. Donaldson, Wistar Institute of Anatomy and Biology.
Caswell Grave, Washington University.
W. E. Garrey, Tulane University.
M. J. Greenman, Wistar Institute of Anatomy and Biology.
George Lefevre, University of Missouri, *Secretary of the Board.*
A. P. Mathews, The University of Cincinnati.
G. H. Parker, Harvard University.
C. R. Stockard, Cornell University Medical College.

TO SERVE UNTIL 1923.

H. C. Bumpus, Brown University.
R. A. Harper, Columbia University.
W. A. Locy, Northwestern University.
Jacques Loeb, The Rockefeller Institute for Medical Research.
George T. Moore, Missouri Botanical Garden, St. Louis.
L. L. Nunn, Telluride, Colo.
W. J. V. Osterhout, Harvard University.
William M. Wheeler, Bussey Institution, Harvard University.

TO SERVE UNTIL 1922.

Cornelia M. Clapp, Mount Holyoke College.
E. G. Conklin, Princeton University.
Ross G. Harrison, Yale University.
Camillus G. Kidder, 27 William Street, New York City.
M. M. Metcalf, Oberlin, Ohio.
William Patten, Dartmouth College.
Jacob Reighard, University of Michigan.
W. B. Scott, Princeton University.

TO SERVE UNTIL 1921.

S. F. Clarke, Williamstown, Mass.
Charles A. Coolidge, Ames Building, Boston, Mass.
C. R. Crane, Woods Hole, Mass., *President of the Corporation.*
Alfred G. Mayor, Carnegie Institution.
C. E. McClung, University of Pennsylvania.
T. H. Morgan, Columbia University.
Erwin F. Smith, United States Department of Agriculture.
E. B. Wilson, Columbia University.

II. ACT OF INCORPORATION

No. 3170.

Commonwealth of Massachusetts.

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our LORD ONE THOUSAND, EIGHT HUNDRED and EIGHTY-EIGHT.

HENRY B. PIERCE,
Secretary of the Commonwealth.

[SEAL.]

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory, in Woods Hole, Mass., at 12 o'clock noon, in each year, and at such meeting the members shall choose by ballot a Treasurer and a Clerk, who shall be, *ex officio*, members of the Board of Trustees, and Trustees as hereinafter provided. At the annual meeting to be held in 1897, not more than twenty-four Trustees shall be chosen, who shall be divided into four classes, to serve one, two, three, and four years, respectively, and thereafter not more than eight Trustees shall be chosen annually for the term of four years. These officers shall hold their respective offices until others are chosen and qualified in their stead. The Director and Assistant Director, who shall be chosen by the Trustees, shall also be Trustees, *ex officio*.

II. Special meetings of the members may be called by the Trustees, to be held in Boston or in Woods Hole at such time and place as may be designated.

III. The Clerk shall give notice of meetings of the members by publication in some daily newspaper published in Boston at least fifteen days before such meeting, and in case of a special meeting the notice shall state the purpose for which it is called.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. The Trustees shall have the control and management of the affairs of the Corporation; they shall present a report of its condition at every annual meeting; they shall elect one of their number President and may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

VI. Meetings of the Trustees shall be called by the President, or by any two Trustees, and the Secretary shall give notice thereof by written or printed notice sent to each Trustee by mail, postpaid. Seven Trustees shall constitute a quorum for the transaction of business. The Board of Trustees shall have power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient.

VII. The President shall annually appoint two Trustees, who shall constitute a committee on finance, to examine from time to time the books and accounts of the Treasurer, and to audit his accounts at the close of the year. No investments of the funds of the Corporation shall be made by the Treasurer except approved by the finance committee in writing.

VIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be given to the Boston Society of Natural History, or some similar public institution, on such terms as may then be agreed upon.

IX. These By-Laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-Laws will be acted upon.

X. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

IV. THE TREASURER'S REPORT

Marine Biological Laboratory Balance-Sheet, December 31, 1920.

Assets.

Cash:		
In bank	$ 572.52	
Petty cash fund	200.00	$ 772.52
Notes receivable (secured)		1,000.00
Accounts receivable		11,706.37
Inventories:		
Supply department	$ 15,796.63	
Biological Bulletin	3,995.02	19,791.65
Investments:		
Securities (Schedule I.)	$ 11,697.43	
Cash (Schedule II.)	712.23	12,409.66
Stock in General Biological Supply House, Inc.		12,500.00
Gansett property account	$ 19,739.27	
Less mortgage	12,132.01	7,607.26
Educational Plant (Schedule III.): Land	$ 95,856.14	
Buildings	191,301.09	
Equipment	89,110.40	
	$376,267.63	
Less reserve for depreciation	34,799.48	341,468.15
Deferred charges:		
Prepaid insurance		3,832.17
Items in suspense		35.85
		$411,123.63

Liabilities.

Accounts payable		$ 4,631.49
Notes payable:		
Falmouth National Bank	$ 5,000.00	
Issued in part payment of shares of General Biological Supply House, Inc. purchased	2,500.00	7,500.00
Accrued charges (estimated)		1,000.00
Trust funds	$ 10,409.66	
Note payable issued on account of purchase of reserve fund securities	2,000.00	12,409.66
		$ 25,541.15

Balancing account:			
Balance, January 1		$359,923.00	
Inventories and sundry capital expenditures set up during year		22,996.65	
Special donations		8,500.00	
Appropriation from Library Fund for purchase of books		813.91	
		$392,234.46	
Deduct, net loss on mess hall and equipment destroyed by fire March 17, 1920	$6,041.33		
Deduct, excess of expenses for year	610.65	6,651.98	385,582.48
			$411,123.63

MARINE BIOLOGICAL LABORATORY, INCOME-AND-EXPENSE FOR YEAR ENDED DECEMBER 31, 1920

	Expense	Income	Loss	Gain
Administration expenses	$ 9,114.98		$ 9,114.98	
Bar Neck property expenses	184.00		184.00	
BIOLOGICAL BULLETIN and annual dues	4,891.86	3,105.75	1,786.11	
BIOLOGICAL BULLETIN, additional expenses for 1919	886.72		886.72	
Carpenter department	909.38	22.90	886.48	
Chemical department	1,647.58		1,657.58	
Dormitories	2,219.51	2,234.18		14.67
Instruction	6,615.36	5,960.00	655.36	
Interest on notes payable	126.55		126.55	
Janitor's house expenses	3.31		3.31	
Lectures, evening	28.22		28.22	
Lectures, philosophical	100.00		100.00	
Library department	2,018.95		2,018.95	
Maintenance, buildings and grounds	6,057.05		6,057.05	
Mess	23,214.76	23,476.72		261.96
New laboratory expenses	2,820.61		2,820.61	
Newman Cottage	49.61		49.61	
Pumping station	228.66		228.66	
Research department	2,571.15	4,450.00		1,878.85
Sundry expense and income	946.27	7,030.75		6,084.48
Supply department	37,672.24	43,662.74		5,990.50
Truck	790.87		790.87	
Total current expenses	$103,097.64		$27,385.06	
Total current income	89,943.04	$89,943.04	14,230.46	$14,230.46

		Expense	Income	Loss	Gain
Excess of expenses		$13,154.60		$13,154.60	
Reserve for depreciation		7,270.69			
Bad accounts written off		245.36			
		$20,670.65			
Donations:					
Friendship Fund	$20,000.00				
Other	60.00	20,060.00			
Balance to balancing account		$610.65			

MARINE BIOLOGICAL LABORATORY, SUMMARY OF INVENTORY OF LAND, BUILDINGS, AND EQUIPMENT, DECEMBER 31, 1920.

A. Land, January 1, 1920		$ 95,856.14
B. Buildings, January 1, 1920	$174,713.44	
Permanent improvements during year:		
New mess hall	14,400.46	
Supply department laboratory	9,205.08	
New laboratory (chemical room extra)	670.94	
Janitor's house	628.41	
New laboratory (third floor improvements)	203.14	
Supply department improvements	14.10	
Mess improvements	27.92	
	$199,863.49	
New refrigerator built in 1919	1,321.57	
	$201,185.06	
Deduct cost of mess hall destroyed by fire March 17, 1920	9,883.97	191,301.09
C. Equipment, January 1, 1920	$ 78,179.37	
Expenditures for new equipment during year:		
Mess hall	6,414.44	
Books and periodicals for library	2,686.16	
New truck	728.14	
Chemical department	267.30	
Carpenter shop	113.87	
Gas machine	108.80	
Supply department	103.50	
New laboratory	57.49	
Main building	11.08	
Dormitories	10.00	
Miscellaneous	56.14	
	$88,736.29	

Cost of books and periodicals purchased in 1916, 1917, 1918, and 1919 set up	3,951.15	
	$92,687.44	
Deduct cost of mess hall equipment destroyed by fire March 17, 1920	3,577.04	89,110.40
		$376,267.63

January 20, 1921.

Mr. D. Blakely Hoar,
161 Devonshire Street,
Boston.

Dear Sir: We have completed our audit of the accounts of the Marine Biological Laboratory for the year ended December 31, 1920, as kept both at your office in Boston and at Woods Hole, and report thereon in the accompanying exhibits and schedules:

Exhibit A—Balance-Sheet as of December 31, 1920.
B—Income-and-Expense for the Year ended December 31, 1920.
Schedule I—Investments (Book Values).
II—Cash Receipts and Payments on Account of Investments.
III—Summary of Inventory of Land, Buildings, and Equipment.
IV—Depreciation Reserve.
V—Supply Department Income-and-Expense for the Year ended December 31, 1920.

We certify that, subject to the comments herewith, the balance-sheet and income-and-expense statement shown in Exhibits A and B are in accordance with the books and correct to the best of our knowledge and belief.

Very respectfully,
Harvey S. Chase & Co.,
Certified Public Accountants.

V. REPORT OF THE LIBRARIAN.

The Library has continued to make a satisfactory growth. The total accessions during the year, not counting periodicals received in parts, were 795 items, of which 175 were received by purchase, 93 by binding, and 514 by gift and exchange. After deducting 13 duplicate volumes removed from the collection, the net increase was 408 volumes and 387 pamphlets, making the total contents of the library on December 31, 1920, 10,243 volumes and 8,532 pamphlets.

The current periodicals and other serial publications, received during the year, comprised 153 titles, of these 83 were received by subscription, 38 in exchange for the BIOLOGICAL BULLETIN, and 32 were gifts. The list of periodicals was increased over the previous year by the addition of 21 titles.

The appropriation of part of the reserve Library Fund made it possible to fill up the gaps in the sets of German periodicals occasioned by the interruption of communication during the war, and also to complete the sets of a number of important journals. As the result of much labor and correspondence the following sets were completed: *Archiv für mikroscopische Anatomie* (except 2 vols.), *Archives de Biologie*, *Biochemical Journal*, *Botanical Abstracts*, *Journal of the New York Botanical Garden*, *Journal of the Royal Microscopical Society*, *New Phytologist* (except 1 vol.), *Parasitology*.

There are still many important sets of journals in the library that are incomplete. And one of the pressing needs of the Laboratory is funds with which to purchase the volumes necessary to complete them.

During the summer the library was in constant use by officers and students of the Laboratory and much of the time of the Assistant Librarian was taken up with assisting readers and attending to the circulation of books. About 1,000 books and periodicals were lent for use outside of the Library.

When the collection was small, the arrangement of the books and pamphlets was a simple matter, and there was little need of a catalogue, except as a matter of record. The collection has now reached so considerable a size that it has become necessary to adopt a definite system of classification and to revise the catalogue so as to make it a useful instrument for finding a book or in searching the literature on a given subject. After careful consideration of the various schemes of classification in general use, it was decided to use, with some modifications, the Dewey Decimal Classification. The main reason for adopting this system was that it seems to be the one most familiar to biologists. During the spring the bound volumes were rearranged and roughly classified according to this system, and during the autumn this work has been continued. The classification is nearly completed, and the corresponding numbers have been placed on the books and on the cards in the catalogue. It is hoped that this work will greatly facilitate the use of the library during the coming summer.

The old arrangement of the pamphlets, alphabetically by authors, has been retained, and in order to aid in finding them, numbers have been assigned to them and have been placed on the catalogue cards; the Cutler notation being used for this purpose.

For the bound volumes a new catalogue has been started on the dictionary principle, author, title and subject entries being arranged in one alphabet.

Much work has still to be done, but it is hoped that the books and the catalogue may ultimately be arranged so as to make the resources of the Library as quickly and easily available as possible.

Another important aim for the future is to complete the sets of important periodicals, and to gather together a complete set of the reprints of investigators, so that the library will fully represent the work done directly or indirectly in the Laboratory. And finally it is hoped that it may be possible to develop the Library symmetrically, so that each branch of Biology may be equally well represented on its shelves.

In conclusion, grateful acknowledgment should be made of the many generous gifts to the Library. Gifts of money have been received from Dr. M. M. Metcalf, $50.00 toward the purchase of volumes of periodicals to complete sets; and from Dr. A. G. Mayor, $10.00 for subscriptions to current journals.

Books have been received from Dr. Marianna L. Herwerden, Dr. E. G. Spaulding, Dr. Dunn, Dr. A. R. Moore, Rev. Mr. Tingley, Mrs. Charles R. Crane, Dr. F. R. Lillie, Dr. Christine Ladd-Franklin, Dr. Otto C. Glaser, the New York Botanical Garden, and others.

ROBERT P. BIGELOW,
Librarian.

VI. THE DIRECTOR'S REPORT.

January 1, 1921.

TO THE TRUSTEES OF THE MARINE BIOLOGICAL LABORATORY.

Gentlemen: I beg to present herewith a report of the thirty-third session of the Laboratory for the year 1920:

On March 17, 1920, the dining hall, kitchen and laundry of the Laboratory were entirely destroyed by a fire which started in the machine shop of the Bureau of Fisheries across the street, and was blown by a strong west wind directly on to our buildings. Although the fire started at 4.30 A.M. the entire town turned out, and by hard work succeeded in saving the other buildings of the Laboratory. A meeting of the members of the Board of Trustees resident in New York and vicinity was hurriedly called on March 20th at the home of Mr. Crane, and voted that "the officers of the Laboratory be empowered to make necessary arrangements to replace the buildings and equipment destroyed by fire, enlarging the same if deemed advisable; and that the Director and Treasurer be given power to borrow the funds necessary for these purposes." A waiver of notice of time and place of this meeting was then sent out and was signed by all members of the Board.

Dr. Drew took charge of the building operations, and, in spite of the unprecedently bad conditions of the market both for labor and materials, he succeeded with the loyal assistance of the Laboratory crew in getting the buildings and equipment ready for opening at the regular time. As the amount of insurance had remained at pre-war rates, a very heavy net loss resulted, which is recorded in the Treasurer's report.

This accident resulted in the interruption of construction of the laboratory of the Supply Department, referred to in my previous report, which was under way at the time, and in diversion of the funds set apart for this purpose. In this emergency Mr. Crane again came to our assistance and furnished the funds needed for the completion of the Supply Department laboratory, the building of which was resumed in the late summer, and is now in process of completion.

The number of colleges, universities and other institutions that coöperate with the Laboratory by subscribing for research rooms and for investigators' or students' tables continues to grow. In 1915 there were 37; 1916, 40; 1917, 38: 1918, 33; 1919, 48; 1920, 52. (See appended list.)

The attendance at the Laboratory was again so large as to tax the capacity of the buildings to the uttermost. The tabular view of attendance (p. 26) shows a total attendance of 256 of whom 120 were students in courses. It was felt that the student laboratories had been unduly crowded last year, especially in embryology and physiology, and a number of student applications were therefore refused, and this explains the slightly smaller student attendance in 1920 compared with 1919. The number of investigators was 136 in 1920, 134 in 1919.

It was felt to be necessary to increase research space for another year, and it was therefore decided to complete the unfinished half of the third floor of the brick building hitherto used for storage. Aid for this work came from the Eli Lilly Co., of Indianapolis, through Dr. G. H. A. Clowes of their research laboratory, who had been carrying on biochemical investigations at the Laboratory for several years. Later the Nela Research Laboratory of the National Lamp Works of the General Electric Co. entered into a similar arrangement through Dr. R. S. Lillie, who was appointed biologist in their organization. By the terms of agreements entered into with these companies they will contribute funds to complete for their own use definitely assigned sections of the new research space, and pay the usual annual fees for their workers at the Laboratory, which on its part agrees to furnish the usual assistance rendered to research workers at the

Laboratory; these agreements run for a period of ten years. The Eli Lilly Co. rooms will be specially equipped for biochemistry, and the Nela Research Laboratory rooms for biophysics, especially radiation work. Thus experience of the special needs in these subjects will be gained in advance of the contemplated erection of a special laboratory for biochemistry and biophysics. The greater part of the new space is involved in these assignments, but other space is also thereby released, making a net gain to the Laboratory of five research rooms.

Additional research rooms can also be secured by the temporary occupation of a portion of the new Supply Department Laboratory, so that accommodations will be more ample for 1921. The relief must, however, be very temporary, and accordingly comprehensive plans for the future development of the Laboratory were studied by members of the Board of Trustees during the summer.

It was decided that any new construction should be part of a plan calculated to meet the needs of the present generation of biologists, and accordingly Mr. Charles A. Coolidge was requested, and kindly consented, to prepare such plans. The obvious immediate needs call for two new buildings, a laboratory building specially equipped for biochemistry and biophysics, and a building for library and administration purposes containing also an auditorium. Future development will require an additional laboratory unit, and this also was included in the plans.

The first two of these buildings should be constructed immediately. As regards the first: not only is present research space inadequate, but, as noted in the last report, the present wooden buildings are not well adapted to the more rigorous needs of modern experimental research. The new laboratory would be fully occupied at once by workers transferred from the old buildings who require the special facilities proposed, and this would leave more space in the old buildings for those whose work has other technical requirements. This building is the one approved by the National Research Council as one of their projects, as related in the report for 1919.

A committee was appointed by the Trustees to consider the internal plans of this building in consultation with Mr. Coolidge. The members of this committee are C. E. McClung (chairman), G. H. A. Clowes, R. S. Lillie, A. P. Mathews and W. J. V. Osterhout. Plans have been drawn up after much study, making the building a wing of the present brick research laboratory extending to the west. It will include about 36 research laboratories and rooms, five dark rooms and laboratories, refrigeration and constant temperature rooms, and space for storage. Some of the research laboratories and rooms will have a strictly biological equipment, others a biochemical equipment, others again biophysical equipment; and it is proposed to interrelate the various types for the most effective coöperation. It is expected that the facilities will be adequate for the most exacting kinds of physical and chemical work adapted to biological analysis.

The second building will extend to the north of the proposed new laboratory and will be connected with it by a corridor. It will be primarily a library building, calculated to be sufficient for the library development of many years to come, and capable of expansion if needed. Administration offices, committee rooms and a demonstration hall will occupy the first floor; and an auditorium will be built out to the east (rear) with access from the demonstration hall. The facilities of this building are greatly needed at the present time. The present administration offices are too small, inconvenient for those who use them, and a source of distraction in the research building in which they are located. The present library rooms are already well filled, and do not provide space for much growth. The present lecture hall is so inadequate that the evening lectures are usually uncomfortably crowded, and attendance at them is discouraged. The committee in charge of this building is G. A. Drew (chairman), R. P. Bigelow, M. J. Greenman, George Lefevre and G. H. Parker.

The third proposed unit will be a laboratory building forming a wing of the present brick laboratory, extending to the east and balancing the proposed west wing. It is not anticipated that it will be needed for a few years yet.

The funds for these buildings and for their maintenance remain to be secured. The chairman of the Division of Biology and

Agriculture of the National Research Council is giving the project for the laboratory of biochemistry and biophysics unremitting personal attention, and other officers of the Council are interesting themselves in the matter as one of their chief projects. Building costs are, however, at their maximum just now, and it is doubtful that any who might be disposed to contribute to these buildings would consider it wise to undertake construction until there has been a considerable reduction of prices. In the meantime, if representatives of all of the 50 or more coöperating universities, colleges and research organizations, and of the industrial research laboratories interested would unite their efforts, the way should be open soon for the construction of the first of these units. The Library, Administration and Lecture Hall building should appeal to a still wider circle, including those interested in the development of Woods Hole as a summer home.

It is perhaps not generally recognized to what an extent costs of operation have increased during the last few years: in 1918 the total current expenditures of the Laboratory amounted to $69,687.14, in 1919 to $88,452.64 and in 1920 to $103,097.64. The current *earned* income for the same years was $54,282.05 (1918), $80,307.57 (1919) and $89,943.04 (1920). This period was one of rapidly mounting costs in supplies of all kinds and in labor;[1] it is not to be expected that 1921 will show an appreciable increase over 1920; indeed, it is to be hoped that some saving can be effected through the fall in prices. It is a matter for congratulation that the various earning departments of the Laboratory were able to keep up with rising prices so that the excess of expenses over income, which is covered by donations, was $7,300.02 less in 1919, and $2,251.49 less in 1920, than in 1918. The Supply Department is largely responsible for this good showing, having increased its income from $22,608.04[2] in 1918 to $43,662.74 in 1920, while its expenses increased during the same period from $25,611.73[2] in 1918 to $37,672.24 in 1920.

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[1] The costs of salaries and wages in 1918, 1919 and 1920 were $33,630.06, $40,307.28 and $54,097.67 respectively.

[2] The system of accounting for the Supply Department was changed in 1919, and the figures given for 1918 are arrived at by applying the 1919 system to the figures given in the treasurer's report for 1918.

Thus an actual loss in 1918 was converted into a reasonable profit both in 1919 and 1920. But all departments show increase of expense, and all earning departments increase of income.

The financial difficulties of 1920 came not from the cost of ordinary operations, but from the losses incident to the fire, and the increased insurance which it was felt necessary to place on the buildings and equipment, and from other capital expenditures. The cost of replacing the mess hall and equipment amounted to $20,814.90 in 1920. The insurance received on the old mess hall and contents was $6,318.54. The net expenditure on this item was therefore $14,496.36. The cost of new insurance was $4,194.72, and, although this covers three to five years insurance, the payments had to be made during the year. Expenditures on the new Supply Department Laboratory exceeded Mr. Crane's special gift by $705.08 during the year. $2,500.00 was invested in new stock of the General Biological Supply House. These expenditures, amounting to $21,896.16, will explain how a cash balance of $14,277.69 on December 31, 1919, was reduced to $772.52 on December 31, 1920, and why it was necessary to borrow $5,000.00 from the Falmouth National Bank. With the cessation of such capital expenditures the current income is adequate to carry all ordinary current expenditures.

Since 1896 the charge for tuition in each of the courses has been $50.00. In 1920 the cost of instruction was $6,615.36 and the income $5,960.00. The salaries paid the instructors, though somewhat increased in the last two years, have been kept consistently low. There are no charges for use of the boats or for equipment, rent, or depreciation. Applications for admission to courses have exceeded accommodations in the last two years. As our Laboratory is primarily a research institution, it is believed that instruction should be self-supporting and of a rigorously professional type. It was therefore decided to raise the fee for each course of instruction to $75.00 beginning 1921. It is hoped that the increase will not debar any who are professionally interested; a hindrance that will exclude any not so interested will not be harmful to the research activities of the Laboratory.

There are appended as parts of this report, lists of the staff and of investigators and students for 1920, a tabular view of attendance from 1916–1920; also lists of the coöperating and subscribing institutions, of the evening lectures and of membership of the Corporation.

1920.

THE STAFF

Frank R. Lillie, *Director*, Professor of Embryology, and Chairman of the Department of Zoölogy, The University of Chicago.

Gilman A. Drew, *Assistant Director*, Marine Biological Laboratory.

ZOÖLOGY.

I. INVESTIGATION.

Gary N. Calkins, Professor of Protozoölogy, Columbia University.

E. G. Conklin, Professor of Zoölogy, Princeton University.

Gilman A. Drew, Assistant Director, Marine Biological Laboratory.

George Lefevre, Professor of Zoölogy, The University of Missouri.

Frank R. Lillie, Professor of Embryology, The University of Chicago.

C. E. McClung, Professor of Zoölogy, University of Pennsylvania.

T. H. Morgan, Professor of Experimental Zoölogy, Columbia University.

E. B. Wilson, Professor of Zoölogy, Columbia University.

II. INSTRUCTION.

W. C. Allee, Professor of Biology, Lake Forest College.

Robert Hall Bowen, Instructor in Zoölogy, Columbia University.

J. A. Dawson, Dalhousie University.

R. T. Hance, Instructor in Zoölogy, University of Pennsylvania.

C. L. Parmenter, Instructor in Zoölogy, University of Pennsylvania.

Christianna Smith, Instructor in Zoölogy, Mount Holyoke College.

J. P. Visscher, Graduate Student, Johns Hopkins University.

Donnell B. Young, Instructor in Biology, Dalhousie University.

PROTOZOÖLOGY.

I. INVESTIGATION.

(*See Zoölogy.*)

II. INSTRUCTION.

Gary N. Calkins, Professor of Protozoölogy, Columbia University.

Louise H. Gregory, Assistant Professor of Zoölogy, Barnard College.

EMBRYOLOGY.

I. INVESTIGATION.

(*See Zoölogy.*)

II. INSTRUCTION.

David H. Tennent, Professor of Biology, Bryn Mawr College.

Charles G. Rogers, Professor of Comparative Physiology, Oberlin College.

Hubert B. Goodrich, Associate Professor of Zoölogy, Wesleyan University.

Benjamin H. Grave, Professor of Biology, Wabash College.

Elizabeth A. Smith, Assistant Professor of Zoölogy, University of Wisconsin.

PHYSIOLOGY.

I. INVESTIGATION.

Albert P. Mathews, Professor of Biochemistry, The University of Cincinnati.

Ralph S. Lillie, Professor of Biology, Clark University.

Harold C. Bradley, Assistant Professor of Physiological Chemistry, University of Wisconsin.

II. INSTRUCTION.

Ralph S. Lillie, Professor of Biology, Clark University.

Walter E. Garrey, Professor of Physiology, Tulane University.

Frank P. Knowlton, Professor of Physiology, Syracuse University.

R. A. Spaeth, School of Public Health, Johns Hopkins University.

PHILOSOPHICAL ASPECTS OF BIOLOGY AND ALLIED SCIENCES.

LECTURER.

Edward G. Spaulding, Professor of Philosophy, Princeton University.

BOTANY.

I. INVESTIGATION.

S. C. Brooks, Associate Professor of Physiology and Biochemistry, Bryn Mawr College.

Edward M. East, Professor of Experimental Plant Morphology, Harvard University.

Robert A. Harper, Professor of Botany, Columbia University.

E. Newton Harvey, Assistant Professor of Physiology, Princeton University.

Winthrop J. V. Osterhout, Professor of Botany, Harvard University.

II. INSTRUCTION.

Ivey F. Lewis, Professor of Biology, University of Virginia.

William Randolph Taylor, Instructor in Botany, University of Pennsylvania.

Conrad Zirkle, Instructor in Botany, University of Virginia.

LIBRARY.

Robert P. Bigelow, Librarian and Associate Professor of Zoölogy and Parasitology, Massachusetts Institute of Technology. Librarian,

Priscilla B. Montgomery (Mrs. Thomas H. Montgomery, Jr.). Assistant Librarian.

CHEMICAL SUPPLIES.*

Oliver S. Strong, Associate Professor of Neurology, Columbia University, New York City, Chemist.

SUPPLY DEPARTMENT.

G. M. Gray, Curator.

John J. Veeder, Captain.

E. M. Lewis, Engineer.

A. W. Leathers, Head of Shipping Department.

A. M. Hilton, Collector.

J. McInnis, Collector.

F. G. Gustafson, Collector in Botany.

Ruth Winkley, Clerk.

F. M. MacNaught, Business Manager.

Herbert A. Hilton, Superintendent of Buildings and Grounds.

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* Compound microscopes, with two oculars and two objectives, rack and pinion and fine adjustment, may be rented for the season at $7.00 each, provided notice is received by the Business Manager not later than June 15. Microscopes are not otherwise supplied by the Laboratory. See under Department of Chemical Supplies (p. 29).

2. STUDENTS AND INVESTIGATORS—1920

INDEPENDENT INVESTIGATORS—Zoölogy.

Addison, William H. F., Professor of Normal Histology and Embryology, University of Pennsylvania.

Allee, Warder C., Professor of Biology, Lake Forest College.

Altenburg, Edgar, Instructor in Biology, Rice Institute.

Amberson, William R., Graduate Fellow in Biology, Princeton University.

Anderson, Ernest G., Carnegie Institution, Cold Spring Harbor.

Bigelow, Robert P., Associate Professor of Zoölogy and Parasitology, Massachusetts Institute of Technology.

Bishop, George H., Instructor in Zoölogy, Northwestern University.

Breitenbecker, Joseph K., Assistant Professor of Biology, Western Reserve University.

Budington, Robert A., Professor of Zoölogy, Oberlin College.

Calkins, Gary N., Professor of Protozoölogy, Columbia University.

Carothers, E. Eleanor, University of Pennsylvania.

Carpenter, Frederic W., Professor of Biology, Trinity College.

Carroll, Mitchel, Professor of Biology, Franklin and Marshall College.

Chambers, Robert, Assistant Professor of Anatomy, Cornell University Medical College.

Charlton, Harry H., University of Missouri.

Clapp, Cornelia M., Professor Emeritus of Zoölogy, Mount Holyoke College.

Clark, Eliot R., Professor of Anatomy, University of Missouri.

Clark, Eleanor L., Columbia, Mo.

Copeland, Manton, Professor of Biology, Bowdoin College.

Danchakoff, Vera, Assistant Professor of Anatomy, College of Physicians and Surgeons.

Dawson, James A., Professor of Biology, Dalhousie University.

Dederer, Pauline H., Associate Professor of Zoölogy, Connecticut College.

Dodds, Gideon S., Assistant Professor of Histology, West Virginia University.

Drew, Gilman A., Assistant Director, Marine Biological Laboratory, Woods Hole, Mass.

Glaser, Otto C., Professor of Biology, Amherst College.

Goodrich, Hubert B., Associate Professor of Zoölogy, Wesleyan University.

Grave, Benjamin H., Professor of Biology, Knox College.

Grave, Caswell, Professor of Zoölogy, Washington University.

Gregory, Louise H., Assistant Professor of Zoölogy, Barnard College.

Hance, Robert T., Instructor in Zoölogy, University of Pennsylvania.

Harvey, Ethel Browne, Princeton, N. J.

Heilbrunn, Lewis V., Instructor, University of Michigan.

Jacobs, Merkel H., Assistant Professor of Zoölogy, University of Pennsylvania.

Jennings, Herbert S., Professor of Zoölogy, Johns Hopkins University.

Just, Ernest E., Professor of Zoölogy, Howard University.

Kindred, James E., Assistant Professor of Biology, Western Reserve University.

Knower, Henry McE., Professor of Anatomy, University of Cincinnati.

LANGE, MATHILDE M., U. S. Dept. of Agriculture, New York City.
LEFEVRE, GEORGE, Professor of Zoölogy, University of Missouri.
LEWIS, WARREN H., Research Associate, Carnegie Institution of Washington.
LEWIS, MARGARET R., Collaborator, Carnegie Institution of Washington.
LILLIE, FRANK R., Chairman, Department of Zoölogy, University of Chicago.
MALONE, EDWARD F., Professor of Histology, University of Cincinnati.
MAST, SAMUEL O., Professor of Zoölogy, Johns Hopkins University.
McCLUNG, CLARENCE E., Director of Zoölogical Laboratory, University of Pennsylvania.
METZ, CHARLES W., Research Associate, Carnegie Institution, Cold Spring Harbor.
MORRILL, CHARLES V., Assistant Professor of Anatomy, Cornell University Medical College.
MULLER, HERMANN J., Instructor, Columbia University.
NEAL, HERBERT V., Professor of Zoölogy, Tufts College, Mass.
OBRESHKOVE, VASIL, Instructor, Syracuse University.
PARKER, GEORGE H., Professor of Zoölogy, Harvard University.
PARMENTER, CHARLES L., Instructor, University of Pennsylvania.
PATTEN, WILLIAM, Professor of Biology, Dartmouth College.
POWERS, EDWIN B., Instructor in Zoölogy, University of Nebraska.
ROGERS, CHARLES G., Professor of Comparative Physiology, Oberlin College.
ROOT, FRANCIS M., Associate in Med. Entomology, School of Hygiene and Public Health, Johns Hopkins University.
SAGUCHI, SAKAE, Professor of Anatomy, Medical School, Kanazawa, Japan.
SMITH, ELIZABETH A., Assistant Professor of Zoölogy, University of Wisconsin.
SPEIDEL, CARL C., Adjunct Professor of Anatomy, University of Virginia.
STOCKARD, CHARLES R., Professor of Anatomy, Cornell University Medical College.
STRONG, OLIVER S., Associate Professor of Neurology, Columbia University.
TAYLOR, CHARLES V., University of California.
TENNENT, DAVID H., Professor of Biology, Bryn Mawr College.
TRACY, HENRY C., Professor of Anatomy, University of Kansas.
WHITE, GERTRUDE W., Instructor, Carnegie Institute of Technology.
WIEMAN, HARRY L., Professor of Zoölogy, University of Cincinnati.
WOOD, F. E., Professor of Biology, Illinois Wesleyan University.
WOODWARD, ALVALYN E., Amherst College.
ZELENY, CHARLES, Professor of Zoölogy, University of Illinois.

BEGINNING INVESTIGATORS—Zoölogy.

AGERSBORG, H. P. K., Assistant in Zoölogy, Columbia University.
BOWEN, ROBERT H., Instructor in Zoölogy, Columbia University.
COHEN, BARNETT, Student, Yale University.
DEMCREC, MILISLAV, Assistant, Carnegie Institution of Washington.
DRIVER, CHARLES S., Columbia University.
GUTHRIE, MARY J., Demonstrator, Bryn Mawr College.
HARRAH, EZRA C., University of Illinois.
HIBBARD, HOPE, Fellow in Biology, Bryn Mawr College.
HINRICHS, MARIE A., Laboratory Assistant, University of Chicago.
HUETTNER, ALFRED F., Assistant in Zoölogy, Columbia University.
JOHNSON, HENRY H., JR., Assistant, Columbia University.

King, Robert L., University of Pennsylvania.
Kirk, Winifred, Technician in Histology, Cornell University Medical College.
Lancefield, Donald E., Assistant, Columbia University.
Lancefield, Rebecca C., Assistant, Carnegie Institution.
Mason, Eleanor D., Assistant, Carnegie Institution.
Moses, Mildred S., Assistant, Carnegie Institution.
Nute, Bertha E., Teacher of Science, Hastings-on-Hudson, N. Y.
Page, Irvine A., Chemist, Eli Lilly & Co., Indianapolis, Ind.
Richter, Maurice N., Student, College of Physicians and Surgeons.
Romer, Alfred S., Columbia University.
Seidlin, Samuel M., Student, College of Physicians and Surgeons.
Smith, Christianna, Instructor, Mount Holyoke College.
Swett, Francis H., Assistant. Yale University.
Visscher, J. Paul, Instructor, Johns Hopkins University.
Warren, Herbert S., Graduate Student, Columbia University.
Williams, Dorothy E., Laboratory Assistant, Mount Holyoke College.
Young, Donnell B., Acting Professor of Biology, Dalhousie University.
Young, Helen D., Research Assistant, Columbia University.

INDEPENDENT INVESTIGATORS—Physiology.

Bradley, Harold C., Professor of Physiological Chemistry, University of Wisconsin.
Brooks, Sumner C., Biologist, Dept. of Public Health, Washington, D. C.
Brooks, Matilda M., 3809 Yuma St., Washington, D. C.
Clowes, G. H. A., Director and Consulting Chemist, Research Laboratory, Eli Lilly & Co.
Edwards, Dayton J., Instructor in Physiology, Cornell University Medical College.
Fenn, Wallace O., Instructor in Applied Physiology, Harvard Medical School.
Garrey, Walter E., Professor of Physiology, Tulane University.
Harvey, E. Newton, Professor of Physiology, Princeton University.
Hecht, Selig, Assistant Professor of Physiology, Creighton University.
Hooker, Donald R., Associate Professor, Johns Hopkins University.
Irwin, Marian, 46 Shepard St., Cambridge, Mass.
Knowlton, Frank P., Professor of Physiology, Syracuse University.
Lillie, Ralph S., Professor of Biology, Clark University.
Loeb, Jacques, Head of Division of Experimental Biology, Rockefeller Institute.
Loeb, Leo, Professor of Comparative Pathology, Washington University.
Mathews, Albert P., Professor of Biochemistry, University of Cincinnati.
Morgulis, Sergius, Professor of Biochemistry and Physiology, Creighton University College of Medicine.
Porter, Eugene L., Assistant Professor of Physiology, Western Reserve University.
Spaeth, Reynold A., Associate in Physiology, School of Public Health, Johns Hopkins University.
Spaulding, E. G., Professor of Philosophy, Princeton University.
Stehle, Raymond L., Assistant Professor of Physiological Chemistry, Medical School, University of Pennsylvania.
Von Herwerden, Marianne, University of Utrecht, Utrecht, Holland.

BEGINNING INVESTIGATORS—Physiology.

Bachman, Eda, Research Chemist, Eli Lilly & Co.
Baskervill, Margaret L., Graduate Student, Columbia University.
Freiberg, Joseph A., Medical College, University of Cincinnati.
Keith, Lucile G., Research Chemist, Eli Lilly & Co.
Pond, Samuel E., Fellow, Clark University.
Sampson, Myra M., Assistant Professor, Smith College.
Yoshida, Sadao, Professor, Osaka Medical College, Japan.

INDEPENDENT INVESTIGATORS—Botany.

Hazen, Tracy E., Assistant Professor of Botany, Columbia University.
Lewis, Ivey F., Professor of Biology, University of Virginia.
Osterhout, Winthrop J. V., Professor of Botany, Harvard University.
Phillips, Everett F., Agriculturist, U. S. Dept. of Agriculture, Washington.
Schramm, Jacob R., Professor of Botany, Cornell University.
Taylor, William R., Instructor, University of Pennsylvania.
Zirkle, Conway, University of Virginia.

BEGINNING INVESTIGATORS—Botany.

Gluck, Marguerite L., 2010 7th Ave., New York City.
Inman, Ondess L., Harvard University.

1920.

STUDENTS

ZOÖLOGY.

Ball, Ruth J., Assistant in Zoölogy Department, University of Vermont.
Belzer, Katharine, Student, Butler College.
Berry, George P., Princeton University.
Bissonnette, Thomas H., University of Chicago.
Blair, Montgomery, Jr., Student, Princeton University.
Buhrer, Edna M., Student, Goucher College.
Bucknell, Lydia E., Student, Oberlin College.
Cass, Mildred F., Student, Doane College.
Cattell, Quinta, Garrison, N. Y.
Cattell, Ware, Student, Columbia University.
Conklin, Isabel, Student, Smith College.
Conover, L. Lenore, Detroit Normal College, Detroit, Mich.
Coventry, Frances A., Goucher College.
Crozier, Annie J., Hunter College.
Drake, Dorothy, Student, Simmons College.
Easton, Charlotte, Skidmore School of Arts.
Edson, Edith M., 511 Dewey Ave., Rochester, N. Y.
Eimert, Helen K., Student, Oberlin College.
Eliot, Ellen P., Student, Simmons College.
Flanagan, Mary C., Student, Simmons College.

Fruit, Irma B., Student, Knox College.
Graves, Isabelle A., Student, Simmons College.
Green, Wilmoth, Teaching Fellow, Washington University.
Gregory, Elizabeth, Sophie Newcomb College.
Griffin, Mary A., 615 McDonough St., Brooklyn, N. Y.
Hershberger, Ralph E., Student, Heidelberg University.
Hickman, Cleveland P., Assistant in Zoölogy, University of Michigan.
Hickman, Jennings R., Assistant in Zoölogy, University of Michigan.
Hole, Frances L., Student, Doane College.
Hook, Flora E., Head of Biology Department, East Orange High School.
Hurd, Archer L., Student, Wesleyan University.
Johnson, Marie M., Knox College.
Kaan, Helen N., Assistant in Biology, Wheaton College.
Keeler, Margery, 28 Strawberry Hill, Stamford, Conn.
Kidney, Mary L., Teacher, Junior High, Quincy, Ill.
Larson, Jean C., Student, Oberlin College.
Lewis, Clara D., Student, Sophie Newcomb College.
Lyman, Annie K., Student, Mount Holyoke College.
Matter, Katherine E., Student, Oberlin College.
McCollum, Ruth, Student, Oberlin College.
McDowell, Claire, Laboratory Assistant, Mount Holyoke College.
Miller, Minniola O., Student, Connecticut College.
Millikin, Neil, Student, Johns Hopkins University.
Mossman, Alexander H., Amherst College.
Osgood, George W., Student, Tufts College.
Quinnan, Bertha C., Teacher, Pauline Shaw School, Boston.
Potter, Grace M., Student, Mount Holyoke College.
Root, Seraph D., Student, Russell Sage College.
Searcy, Augusta T., Instructor in Biology, University of Alabama.
Shahn, Benjamin H., Student, New York University.
Sheehan, Fred M., Student, Lake Forest College.
Stoll, Harold F., Student, Western State Normal School.
Thorp, Erica, 115 Brattle St., Cambridge, Mass.
Williams, S. Culver, Student, Wesleyan University.
Yolton, Leroy W., Student, Illinois Wesleyan University.
Zoomaya, Ezra B., Student, Lake Forest College.

PROTOZOÖLOGY.

Dietrich, Bertha K., Teacher, Bushwick High School, Brooklyn.
Elliott, Margaret E., Teaching Assistant, Wellesley College.
Farrar, Hilda, Teacher of Biology, East High School, Rochester, N. Y.
Greenleaf, William E., Graduate Student, Yale University.
Helbig, Dorothy M., Hunter College.
Lackey, James B., Mississippi College.
Leiva, Lamberto, Instructor in Parasitology, University of the Philippines.
Lowther, Mrs. Florence deL., Instructor, Barnard College.
Provost, Margaret, Teacher of Biology, Wadleigh High School, New York City.
Sheppard, Alice E., 722 King St., Pottstown, Pa.

Spencer, Clementina S., Assistant Professor, Coe College.
Tubangui, Marcos A., Fellow, University of the Philippines.
Uhlemeyer, Bertha, Assistant, Washington University.
Wallace, Grace N., Mount Holyoke College.
Yocum, Harry B., Assistant Professor, College of the City of New York.

EMBRYOLOGY.

Bean, Raymond J., Instructor, Western Reserve University.
Burns, Robert K., Bridgewater College.
Coldwell, Esther I., Graduate Student, Johns Hopkins University.
Duryea, Arthur W., Student, Dartmouth College.
Fries, Erik F. B., Student, Harvard University.
Graham, Margaret A., Instructor, Hunter College.
Hall-Quest, Shirley K., Clifton, Cincinnati, Ohio.
Hannum, Margaret H., Student, Smith College.
Hermetet, McGreer, Student, Knox College.
Hippolitus, Jennie D., Student, Connecticut College.
Johnson, Helen G., Teacher, Hartford High School.
Johnson, Henry H., Jr., Assistant in Zoölogy, Columbia University.
Ma, P. W., North China Union College.
McClung, Ruth C., Student, Kansas University.
Montanus, John J., Student, Long Island College Hospital.
Paxson, Eleanor M., Student, Swarthmore College.
Pearse, Herman E., Jr., Student, University of Missouri.
Rand, Elizabeth P., Student, Wellesley College.
Robbins, Harriet L., Teacher of Biology, Kansas Wesleyan University.
Simkins, Cleveland S., Harvard University.
Smith, Louise, Instructor in Zoölogy, Smith College.
Stacey, Helen R., Vassar College.
Thompson, Martha L., Teaching Fellow, University of California.
Walters, Mary J., 1633–29th St., N.W., Washington, D. C.
Williamson, Janet A., Instructor in Zoölogy, Mount Holyoke College.
Wondergem, Henry E., University of Rochester.

PHYSIOLOGY.

Chace, Eunice E., Instructor, Smith College.
Crofts, Elizabeth E., Oberlin College.
Gilson, Arthur S., Jr., Student, Harvard University.
Greene, Walter F., Assistant Instructor, Yale University.
Hanna, Leila O., University of Toronto.
Heizer, Pauline, Student, Radcliffe College.
Johnson, George E., Professor of Biology, University of Porto Rico.
Klopp, John W., Swarthmore College.
Kuyk, Margaret P., Professor of Physiology and Hygiene, University of Richmond.
Mall, Mary L., Bryn Mawr College.
Pollak, Marion H., 135 West 87th St., New York City.
Thompson, James T., Wabash College.

Weed, Eleanor H., Vassar College.
Wollf, Nathaniel S., 25 Portsmouth Terrace, Rochester, N.Y.
Yen, Hui C., Oberlin College.

BOTANY.

Amory, Walter, Student, Harvard University.
Chute, Richard, Student, Harvard University.
Dodge, Carroll W., Assistant Professor of Botany, Brown University.
Mader, Vivienne C., Student, Connecticut College.
Mason, Marian, Student, Vassar College.
Osterhout, Anna M., Student, Vassar College.
Rogers, Lenette M., Student, Mount Holyoke College.
Todd, Jessie E., 4539 Jackson Boulevard, Chicago, Ill.

3. TABULAR VIEW OF ATTENDANCE

	1916	1917	1918	1919	1920
Investigators—Total	129	129	93	134	136
Independent:					
Zoölogy	70	63	51	68	69
Physiology	23	23	16	24	22
Botany	7	8	5	7	7
Under Instruction:					
Zoölogy	25	24	16	21	29
Physiology	3	6	3	10	7
Botany	1	5	2	4	2
Students—Total	102	83	69	128	120
Zoölogy	50	46	41	55	56
Protozoölogy	—	—	—	15	15
Embryology	26	16	12	33	26
Physiology	14	13	10	17	15
Botany	12	8	6	8	8
Total Attendance	231	212	162	262	256
Institutions Represented—Total	73	77	72	88	86
By investigators	51	60	49	61	55
By students	45	36	38	62	57
Schools and Academies Represented.					
By investigators	—	2	—	—	1
By students	3	5	—	4	7

4. COÖPERATING AND SUBSCRIBING INSTITUTIONS—1920

- Barnard College
- Bowdoin College
- Bryn Mawr College
- Butler College
- Carnegie Institution of Washington
- Carnegie Institution, Cold Spring Harbor
- Columbia University
- Connecticut College
- Cornell University Medical College
- Creighton University
- Dartmouth College
- Doane College
- Eli Lilly & Co.
- Goucher College
- Harvard University
- Harvard University Medical School
- Hunter College
- Johns Hopkins University
- Knox College
- Lake Forest College
- Mount Holyoke College
- Oberlin College
- Princeton University
- Radcliffe College
- Rice Institute
- Rockefeller Institute for Medical Research
- Simmons College
- Smith College
- Sophie Newcomb College
- Trinity College
- University of Chicago
- University of Cincinnati
- University of Illinois
- University of Kansas
- University of Michigan
- University of Missouri
- University of Pennsylvania

University of Pennsylvania Medical School
University of the Philippines
University of Rochester
University of Toronto
University of Vermont
University of Wisconsin
Vassar College
Washington University
Wellesley College
Wesleyan University
Western Reserve University
Western State Normal School
Wheaton College
Wistar Institute of Anatomy and Biology
Yale University

5. EVENING LECTURES, 1920

Friday, July 2,
Dr. G. H. Parker. . . . . "Activities of Renilla."
Tuesday, July 6,
Dr. C. R. Stockard. . . . . "Structure and Type as Influenced by Rate of Development and Growth."
Friday, July 9,
Dr. Jacques Loeb. . . . . "General' Chemistry and Colloid Chemistry of Proteins."
Tuesday, July 13,
Dr. C. W. Metz. . . . . "Studies on the Relationships of Chromosomes in Different Species of Drosophila."
Friday, July 16,
Dr. R. M. Yerkes. . . . . "The Intelligence of Biologists."
Tuesday, July 20,
Dr. Charles Zeleny. . . . . "Some Factors Controlling Eye-Facet Number in Drosophila."
Friday, July 23,
Dr. Christine Ladd-Franklin. "Theories of Chromatic and Achromatic Sensations."

Tuesday, July 27,
Dr. Henry Laurens ....“The Investigation of the Stimulating Effect of Visible Radiation in an Equal-Energy Spectrum.”

Friday, July 30,
Dr. Raymond Pearl ....“Some Problems in Human Biology.”

Tuesday, Aug. 3,
Dr. Leo Loeb ....“Transplantation and Individuality.”

Friday, Aug. 6,
Dr. Warren H. Lewis ....“The Reactions of Cells in Tissue Cultures.”

Tuesday, Aug. 10,
Mr. W. Lyman Underwood ...“Adventures in the Land of Sunshine.”

VI. MEMBERS OF THE CORPORATION.

1. Life Members.

Allis, Mr. E. P., Jr., Palais Carnoles, Menton, France.
Andrews, Mrs. Gwendolen Foulke, Baltimore, Md.
Billings, Mr. R. C., 66 Franklin St., Boston, Mass.
Carey, Mr. Arthur Astor, Fayerweather St., Boston, Mass.
Clarke, Prof. S. F., Williamstown, Mass.
Conklin, Prof. Edwin G., Princeton University, Princeton, N. J.
Crane, Mr. C. R., Woods Hole, Mass.
Davis, Major Henry M., Syracuse, N. Y.
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STUDIES ON THE PARASITIC WASP, HADROBRACON BREVICORNIS (WESMAEL).

I. Genetics of an Orange-eyed Mutation and the Production of Mosaic Males from Fertilized Eggs.

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Introduction.

In a previous paper (1918) I gave a brief account of the general biological relationships and the method of sex-determination in the parasitic wasp, *Hadrobracon brevicornis* (Wesmael). The experiments there reported were begun in the fall of 1916 and continued through the winter. Difficulties in rearing the host caterpillars in sufficient quantity induced me to abandon the work on the wasp and to concentrate on the host, the Mediterranean flour-moth, which was also being investigated genetically. When these technical difficulties were overcome, the work with *Hadrobracon* was renewed and has been continued since June, 1919. During the summer of 1919, the wasps were bred at the Marine Biological Laboratory, Woods Hole, Massachusetts; during the winter of 1919–20, at Franklin and Marshall College, Lancaster, Pennsylvania; and during the summer of 1920 at the Carnegie Station for Experimental Research, Cold Spring Harbor, Long Island. The work is being continued at St. Stephen's College, Annandale-on-Hudson, New York.

My grateful acknowledgments are due for the hospitality of the Woods Hole Laboratory and of the Carnegie Station at Cold Spring Harbor. I am also indebted for financial aid to the Carnegie Institution of Washington and to the American Association for the Advancement of Science from which I have received two grants for research. Mrs. Whiting has given me valuable assistance in the course of the work.

A type of reproduction in which virgin females produce only males while mated females may produce either males or females has been shown to occur in several groups. Such "male-producing" species are called arrenotokous and it has been supposed that all males arise from unfertilized eggs, females from fertilized. This theory bears the name of Dzierzon (1845) who first applied it to the honey-bee. The males produced by mated females are assumed to develop from eggs into which spermatozoa have not penetrated. Cytological observations showing that males of several arrenotokous species are haploid, while females are diploid tend to corroborate the theory derived from breeding tests.

The only published experimental work on arrenotokous forms showing the method of inheritance of any character difference, other than that of sex, appears to be in crosses of different races of honey-bees. "Criss-cross" inheritance is the rule; the males resemble the mother, while the females inherit characters from both parents. Cross-bred females produce drones showing segregation in expected one to one ratio. From the similarity of these phenomena to sex-linkage, this method of inheritance may be called *sex-linkoid*.

PRESENTATION OF DATA.

(*a*) *Black-eyed Stocks.*—The results in reference to sex-determination published in my previous paper (1918) showed that the stock of *Hadrobracon brevicornis* obtained in Philadelphia at that time was arrenotokous. The data may be briefly summarized as follows:

Sixty-one females set with males produced 683 males and 918 females.

Nine females set with males produced 197 males, no females.

Thirty-six virgin females produced 1,133 males, no females.

Impaternate sons were crossed back to their virgin mothers and in this way closely inbred daughters were obtained.

Another stock of *Hadrobracon* was secured in Philadelphia in December, 1917, and three virgin females were isolated, producing in all 244 males. One of these females was later mated to one of her sons and produced 9 males and 3 females.

Three males and one female were caught in Mifflintown, Pennsylvania, May, 1920. From eggs laid by this female there were produced 4 males and 12 females. Seven virgin daughters produced 804 males, and one of these females when later crossed to a son produced 34 males and 5 females.

The Lancaster stock, upon which most of the work with *Hadrobracon* has been done, was derived from a single parasitized caterpillar found in my cultures of the Mediterranean flour-moth at Lancaster in June, 1919. It is highly probable that the caterpillar was stung by a single female which came in from some bakery or grocery store near-by, as I had no cultures of *Hadrobracon* at that time. Seven females were reared, showing that the mother had evidently mated. These females, isolated without males, produced only males which were crossed back to their virgin mothers in order to obtain females. This stock has been bred since that time and has shown considerable variation.

It will be necessary to give only summaries of the typical black-eyed Lancaster stock as the data is very extensive and results in reference to other somatic variations and sex-ratio will be published later.

Fifty-three females, isolated as virgin, produced 5,948 males and no females.

Forty females, set with males, produced 3,944 males, no females.

One hundred thirty-five females, set with males, produced 7,270 males and 4,791 females.

We may now give the entire summaries of the black-eyed stocks, including two different stocks from Philadelphia, one from Mifflintown, and one from Lancaster.

From one hundred and thirty-five virgin females there were 9,242 males.

From forty-nine females set with males there were 4,141 males.

From one hundred and ninety-nine mated females there were 8,000 males and 5,729 females.

I feel very certain that among all these wasps there were none of the orange-eyed variety described below. The difference between the typical black-eyed form and orange is so striking and the eyes of the wasp are so large that such a variation could not escape attention. Moreover the material bred was examined with some care as one of the main purposes of the work was to find genetic differences.

(*b*) *The Orange Mutation.*—On March 11, 1920, a female, in all respects typical, was isolated as a virgin. Her progeny consisted of 253 black-eyed males and 1 male with orange eyes. This mutant occurred in the first bottle in which the mother had been kept for a period of seven days. The count from this bottle on March twenty-seventh numbered 46 including the mutant. The cultures were at that time developing in the incubator at thirty degrees centigrade at which temperature the period from oviposition to eclosion is about ten days. The mutant may have emerged a few days before the count was made. In any case the egg from which he came was laid within the first seven days of the adult life of the mother which lived until April 1. The mutation may have occurred during the development of a single egg; at least it could not have taken place many cell generations before maturation.

The compound eyes of the mutant were rather light orange. They became slightly darker with age. He was kept alive until May tenth and was mated to various females. "Orange" eyes as they have appeared in later generations are not necessarily orange. They are found to vary from very light cream to deep red and from yellowish orange to pink. No trace of a black fleck such as occurs in the eye of *Drosophila* has been found. The peripheral ommatidia appear black, but this is due, as sections show, to black pigment in the underlying and adjacent integument. Black pigment is also entirely lacking from the ocelli, although here again it occurs in the adjacent integument. In the typical wasp the eyes, both compound and simple, are always jet black. Variations in orange are not considered in the present paper which is concerned only with the difference between black and orange.

(*c*) *Sex-linkoid Inheritance of Orange.*—The mutant was crossed successfully with six black females and offspring were reared as follows:

Culture No.	Black Males.	Black Females.
178	81	94
179	49	65
180	73	87
182	39	68
183	162	67
185	1	2
Total... 6	405	383

By expectation from sex-linkoid inheritance the daughters should carry orange, the sons should be without it. The dominance of black was complete. Four isolated daughters bred as follows:

Culture No.	Origin.	Black Males.	Orange Males.
194	178	94	129
198	178	90	91
199	180	16	25
202	179	68	81
Total...4		268	326

The excess of orange over the expected one to one ratio indicates that orange is not deficient in viability.

Eight daughters that had mated with their brothers bred as follows:

Culture No.	Origin.	Black Males.	Orange Males.	Black Females.
188	178	48	32	77
189	"	30	43	51
190	"	26	32	70
191	"	23	14	49
192	"	65	68	58
196	"	26	26	50
197	"	37	21	63
212	183	2	3	7
Total 8		257	239	425

The failure of orange to appear in the females is to be expected from sex-linkoid inheritance.

The mutant orange male was crossed successfully to three of his heterozygous daughters and produced offspring as follows:

Culture No.	Origin.	Black Males.	Orange Males.	Black Females.	Orange Females.
193	178	52	66	27	36
200	180	81	75	13	16
201	179	88	102	4	7
Total ..3		221	243	44	59

Here in accordance with expectation the orange-eyed females appeared for the first time. In eye color they resembled the orange males and in later generations have shown apparently the same range of variability. The numerical deficiency of females as compared with their brothers is in this case probably due to the age of the mutant when mating occurred and a consequent scarcity of spermatozoa.

The mutant was set with four of his orange granddaughters, but only a single female was obtained. The record of offspring is as follows:

Culture No.	Origin.	Orange Males.	Orange Females.
209	193	211	0
211	193	120	0
312	201	136	1
313	201	88	0

The single orange female from mating 312 was crossed to one of her brothers and produced 35 orange males and 47 orange females. Twenty-five of these orange females, isolated as virgin, produced 2,549 orange males. By back-crossing some of these males to their virgin mothers, orange females were produced and a stock obtained which has since bred true.

Further crosses involving orange may now be summarized.

Twenty-nine black females from various homozygous black stocks crossed with various orange males, produced all black, 1,369 males and 665 females.

Two pairs of heterozygous females by black males produced 28 black males, 26 orange males, and 39 black females.

Twenty-six heterozygous females derived from various crosses of black by orange produced only males, 967 black and 996 orange.

Seven other heterozygous females crossed with orange males produced 170 black males, 151 orange males, 82 black females and 99 orange females.

Thirty virgin orange females derived from various sources produced 2,996 orange males, no females.

Twenty-eight orange females set with males produced 3,365 orange males, no females.

Forty-seven orange females, from various sources mated with orange males likewise from various sources, but not from the cross of orange female by black male, produced only orange, 2,333 males and 1,002 females.

The progeny from orange males derived from orange females crossed with black males are summarized below.

The inheritance is thus far seen to be quite in accordance with expectation.

The case must be mentioned of the occurrence of a single orange male in a cross (237) involving only type stock. There were produced 115 black males and 83 black females besides this orange male. This was possibly a second mutant as it is highly improbable that contamination of the culture could have taken place.

(*d*) *The Occurrence of Anomalous Males.*—The cross of black male by orange female remains to be considered. Black daughters and orange sons are to be expected. Thirty-three such crosses have been made and the results are summarized in Table I. Eleven agree with expectation, giving 183 black females and 445 orange males. The other twenty-two, however, produced in addition to 816 black females and 889 orange males, *57 black males.*

It has been shown above that the sons of virgin orange females have always been orange. These anomalous blacks must then have derived their eye color from the sperm nucleus, and consequently must have developed from fertilized eggs.

Anomalous black males and some of their orange brothers were tested by mating to orange females. Table II. shows the results of these tests. Seven black males produced orange daughters only, showing that although their eyes were paternal in origin their gonads were from maternal nuclei. They are therefore mosaics. One of these males, No. 2, was mated successfully to five orange females and sired in all 118 orange daughters.

Five black males produced black daughters only and hence their gonads as well as their eyes were of paternal tissue. One of these, No. 8, was tested with two females.

Eighteen orange males sired orange daughters only. These males were therefore produced in regular manner from unfertilized eggs or, if sperm nuclei entered, they took no part in the formation of eyes or gonads.

One orange male, No. 17, produced 19 black daughters, thus showing that although his eyes were orange his gonads were paternal in origin.

From the foregoing data it is to be noted that, while black females may be heterozygous and thus diploid for the orange locus, males produce one type of sperm only, which may carry either black or orange but not both.

TABLE I.

PROGENIES FROM BLACK MALES BY ORANGE FEMALES.

Culture No.	Black Males.	Orange Males.	Black Females.
205	4	65	41
207	6	67	61
214	1	28	34
219	3	85	67
281		11	14
282	1	35	42
289	3	23	21
340	1	8	58
342	2	39	45
343	6	49	47
344	2	86	77
381		161	38
384	1	62	64
387	1	13	11
388	1	30	34
391	4	42	13
393	3	38	42
394		36	21
395		20	22
396	1	17	15
397	3	42	34
398	2	31	16
399		4	1
400	1	37	22
401		16	8
402	2	34	17
403		13	16
404		26	34
405	4	36	32
407		2	2
408		4	2
409	5	22	23
554		152	25
Total 33	57	1334	999

When pairs of wasps are set together it often happens that the female will refuse to mate and will kick the male away, bending her abdomen down and forward. Her eggs then produce only males as expected. The same male, set with another female or with the same female at another time may succeed in siring

Table II.

Tests of Anomalous Males and Orange Brothers.

Males.			Offspring.		
No.	Origin.	Character.	Orange Males.	Orange Females.	Black Females.
1	219	Orange	81	63	
2a	219	Black	167	28	
2b	"	"	30	24	
2c	"	"	66	24	
2d	"	"	75	35	
2e	"	"	10	7	
3	282	"	83	40	
4	342	Orange	112	58	
5	"	"	10	8	
6	"	"	65	58	
7	343	Black	24		3
8a	344	"	192		2
8b	"	"	58		19
9	"	Orange	4	2	
10	384	"	10	11	
11	"	"	6	14	
12	"	"	11	17	
13	"	"	19	26	
14	387	Black	44	41	
15	388	"	35		1
16	391	"	100	64	
17	"	Orange	67		19
18	"	"	37	14	
19	393	Black	45	1	
20	"	Orange	54	30	
21	"	"	23	34	
22	"	Black	104	65	
23	"	Orange	36	17	
24	"	"	90	48	
25	396	Black	94		1
26	397	Orange	81	73	
27	"	"	173	1	
28	"	Black	191		1
29	"	Orange	87	52	
30	"	"	3	6	
31	409	Black	21	3	
Totals......	18 Orange males		902	532	—
	1 Orange male		67	—	19
	7 Black males		745	332	—
	5 Black males		594	—	27

daughters. Male broods, therefore, do not necessarily mean that the male is sterile. In the case of anomalous males, however, an apparently successful mating frequently results in no daughters or as Table II. shows, in very few, although the female may be highly fertile as indicated by the number of sons produced. Data in reference to this matter are not yet extensive enough for a thorough analysis, and it is therefore thought best to defer publication until later.

Discussion.

(*a*) *Sex-linkoid Inheritance in the Honey-bee.*—Johannes Dzierzon (1845) based his theory of the parthenogenetic origin of drones of the honey-bee, in part at least, upon crosses of various races. Clear evidence of sex-linkoid inheritance in the bee has been recently furnished by Newell (1914) who crossed a yellow Italian queen to a gray Carniolan drone. All the offspring were yellow. The reciprocal cross produced yellow females and gray males. Heterozygous females produced yellow and gray drones in equal numbers.

Many investigators have crossed Italian bees to the black French or German forms. Sex-linkoid inheritance appears to be the rule here also but there are numerous apparent exceptions.

Perez (1879) crossed yellow Italian queen to black French drone and examined three hundred drone offspring. Forty-nine showed markings interpreted as of paternal origin.

Cuénot (1909) crossed black female to yellow male and obtained females, all of which had yellow bands; three hundred black males; twelve males with small amount of yellow; and two males with broad yellow bands.

Inasmuch as drones intergrading between black and yellow occur it is natural to suppose that there is more than one factorial difference between the two races. It has been suggested that those males which were apparently more or less patroclinous might have arisen from eggs laid by hybrid workers, or that the queen might have been of mixed origin. It may be, however, that the intergrading drones were mosaics, comparable to the mosaic males of *Hadrobracon.*

(*b*) *Sex Determination and Parthenogenesis.*—In most animals there appears to be a sex-difference in chromatin content. This varies, from a theoretical minute portion of one chromosome to a clearly visible difference of one or more chromosomes. In the case of haploid parthenogenesis, the male has the haploid number, and reduction of the egg nucleus takes place in a normal manner. This occurs apparently in at least most of the Hymenoptera, in the white fly (Stoll, 1919; Schrader, 1920) and probably in several other forms. Diploid parthenogenesis occurs in many animals. It may result in the production of either males or females, but in Hymenoptera it results in females only, the males being haploid, with the possible exception of the saw flies (Doncaster, 1907). Goldschmidt (1917) has given a convenient classification of types of parthenogenesis.

From the work already done on *Hadrobracon* it appears that parthenogenesis is strictly haploid and therefore arrenotokous. The females are certainly diploid and arise in all cases from fertilized eggs. But it does not therefore follow that all males come from unfertilized eggs, or that such males as arise from fertilized eggs are diploid.

The suggestion has been made that males in Hymenoptera might occur, having the number of chromosomes diploid, except for the sex-chromosome, which might occasionally pass out in the abortive division of the first spermatocyte. Fertilization by the resulting spermatozoön would result in diploid males. In *Hadrobracon* all males, thus far studied, even the anomalous males, appear to be haploid for the orange locus.

(*c*) *The Bearing of Mosaics on Problems of Fertilization.*—An interesting problem in the occurrence of anomalous, mosaic males centers in fertilization. In the physiological sense, fertilization means the stimulation necessary to initiate development. In forms which are normally parthenogenetic, this stimulation is unnecessary and the entrance of the spermatozoön into the egg can hardly be called physiological fertilization. The egg is already fertile before the appearance of the male cell. In the genetic sense, however, fertilization denotes the union of paternal with maternal germ plasm, or amphimixis. In this sense an egg producing an anomalous male is fertilized, for paternal germ plasm is brought into it, although this is not followed by fusion of nuclei, as is usually the case.

In order to explain sex-mosaics or gynandromorphs it has been suggested that a paternal nucleus might fuse with a maternal, the diploid product giving rise to the female parts of the embryo. Male parts are assumed to be derived from haploid nuclei of paternal, maternal or mixed origin. Polyspermy has been suggested by Morgan (1905) to explain the Eugster gynandromorph bees, but Boveri (1915) contended that the male parts were of maternal color and held that a single sperm nucleus had fused with one of the products of parthenogenetic cleavage. Morgan's hypothesis apparently fits the von Engelhardt bees (1914) in which the male parts resembled the father. Cleavage of a single sperm nucleus, however, might be followed by subsequent fusion of one of the products with the female pronucleus and a result would be obtained similar to Morgan's hypothesis of polyspermy. It is also obvious that haploid nuclei might be derived from both parents and the resulting male parts would then be of mixed origin.

Doncaster (1914) has demonstrated the existence of binucleated eggs in *Abraxas;* and it has been suggested that if one of these contained the sex-chromosome and the other lacked it, their simultaneous fusion with two sperm nuclei would result in a gynandromorph.

Morgan and Bridges (1914) have shown that in *Drosophila* gynandromorphs and other mosaics result from dislocation of a sex-chromosome during cleavage or embryonic development. The male parts are here of course diploid for autosomal factors.

Mosaics due to somatic mutation have likewise been reported in several plants and animals.

It is to be noted that the mosaic males reported in the present paper result from the cross of orange female by black male. The reciprocal cross has failed to give any anomalous results. Whether the production of anomalous males is necessarily correlated with the fact that orange comes in from the mother, black from the father; or whether black females may be obtained which will give anomalous results when crossed to orange males, is uncertain. In the former case the orange factorial difference would itself be responsible for the production of anomalous males. In the latter case the production of irregularities would perhaps depend upon a factorial difference other than that of orange. The answers to this question and to others raised in the present paper may be given by investigations now in progress.

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CHROMOSOMES AND THE LIFE CYCLE OF HYDATINA SENTA.[1]

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A reëxamination of the chromosomes of this rotifer seemed desirable owing to the results of some of my early experiments (Shull, '12), in which it was shown that whether one of these rotifers is to be a male-producer or female-producer is irrevocably decided within a period several hours in duration occurring in the course of maturation of the parthenogenetic egg from which the individual develops. Influences acting upon the egg before and after that period were found to be without effect. This result indicated that some very brief event occurring in maturation decided the fate of the female arising from the egg, and it seemed possible that the event might be a change in the number of chromosomes, perhaps comparable to the chromosome change in the aphids and phylloxerans, in which the diminished number in the male is still greater than the haploid. Sharp distinction between male-producers and female-producers is known in the phylloxerans without any difference in the chromosome number (Morgan, '09); but in these insects the time at which the differentiating event occurs is unknown.

A difference in the number of chromosomes between male-producers and female-producers was not excluded by the results of previous studies of the cytology of *Hydatina*. Lenssen ('98) states the number of chromosomes to be 10 or 12 in the female-producing egg. In one passage it is plain that these numbers imply uncertainty as to the correct number. Perhaps uncertainty was intended to be implied by the other passages in which the expression "10 or 12" is used, but there is room in one case to suppose that Lenssen observed sometimes 10, sometimes 12, in different eggs. If such differences existed, they might represent the two types of female. The possibility that Lenssen believed the number of chromosomes to be different in different individuals is heightened by his statement that the reduced number in the egg of the male-producer is "probably five." This would suggest 10 as the diploid number in the male-producer, though maturation phenomena might reduce the haploid number to less than half the diploid.

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[1] Contribution from the Zoölogical Laboratory of the University of Michigan. For aid in the laborious task of preparing the specimens from which this study was made the author is indebted to the trustees of the Bache fund.

Whitney's ('09) studies threw no light on Lenssen's statements, for he was unable to count the chromosomes with accuracy. Using American specimens, he arrived at a diploid number between 20 and 30, the highest number actually counted being 25. There was nothing in the male egg or fertilized egg to render the count more definite, for the number discovered in the male egg ranged from 10 to 14, and in the fertilized egg, during maturation, 14 was the largest number seen.

In view of the uncertainty of these determinations, and of the possibility that the male-producing and female-producing females might differ in their chromosome number, I undertook a reexamination of this difficult material. Notwithstanding the difficulties, I have a fair degree of confidence in the conclusion reached.

Preparation of Material.

Although several fixing agents were used, the specimens that were good enough to study were all fixed in Bouin's fluid. Dehydration was accomplished by the drop method. When the objects had reached 70 per cent. alcohol, they were carefully wrapped in bits of stratum corneum of frog skin, which had shortly before been put into 70 per cent. alcohol, in order to make the objects large enough to imbed easily. The frog skin was held in compact form by fragments of cover glass until the objects were in 96 per cent. alcohol, by which time the skin had hardened so that it would not unroll. Six to eight rotifers were as a rule wrapped in each roll. Sections were all cut 5 μ in thickness. All were stained in iron-hematoxylin. They were also lightly stained with eosin, but not for the purpose of this study. Unfortunately the chromosomes show a strong tendency to collect in masses, so that the number of specimens clear enough to furnish counts was not large.

General Course of Maturation.

Inasmuch as I was primarily interested in the number of chromosomes, in each of the kinds of females, I have made no attempt to render a complete account of maturation. Such facts as have been revealed, even though incidental to the main object, are here recorded. Only that part of the maturation which occurs in the oviduct of the female has been studied. The completion of the process after the egg is laid has not been followed.

About the time when the oöcyte has reached its maximum size and is fairly fixed in form (ellipsoidal), not yielding to the movements of the body of the female, the nucleus rapidly increases in volume. An aster appears in the cytoplasm near it, being readily visible by the practical absence of yolk spherules, although radiations are very indistinct. So plain is the aster that it was invariably used as a guide-post in locating maturation spindles. The spindle is formed within the nucleus. Upon it the chromosomes appear as long slender threads tapering toward both ends (Fig. 1). As the spindle develops the nuclear membrane disappears, so that the spindle is out in the cytoplasm: but there is always a definite space in the cytoplasm in which the spindle is located so that there is no confusion of chromosomes and yolk spherules. With very few exceptions, the spindle, which is near the periphery of the cell, is turned toward the intestine. Exceptions to this rule are more common in male-producers than in female-producers.

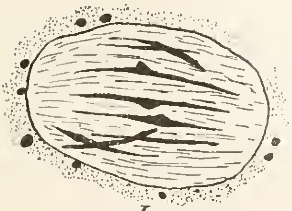
In the female-producing egg the chromosomes arrange themselves on the equator. In this condition the cell appears to remain until the egg is laid, for out of a large number of specimens not one was found that had proceeded beyond that stage. Lenssen ('98), indeed, concluded that the division was never completed; but Whitney ('09) observed the single polar body at the periphery of the segmenting egg.

In the male-producing egg several specimens indicate that the chromosomes meet in pairs on the equator of the spindle. The pairs separate into their components, each group proceeding to the end of the spindle. In this late anaphase the cell appears to remain until the egg is laid, for out of many specimens none was found in later stages.

Number of Chromosomes.

The tendency of the chromosomes to adhere to one another in masses has rendered the determination of their number difficult. Fortunately in my material a number of specimens appear to agree in the number, and this is the largest number found. In such specimens the chromosomes are of approximately uniform size, while in cells showing smaller numbers some chromatin masses are nearly always distinctly larger than others, indicating that the larger bodies are probably compound. From a study of this material I conclude that the diploid number of chromosomes is 12. In female-producing parthenogenetic eggs, the best stage for counting the chromosomes is just before or during the formation of the equatorial plate, because the chromosomes are then well-defined, and are not so crowded as later in the equatorial plate. Such a stage is represented in Fig. 2.

The chromosomes of the male-producing egg can be counted fairly well at several stages. As in the female-producing egg, one favorable stage is just before the formation of the equatorial plate. In the equatorial plate the chromosomes can also be counted, in these eggs, because the chromosomes unite in pairs, hence there is a smaller number of discrete objects, and they are not so crowded. Figure 3 shows distinctly six pairs. In this figure the equatorial plate is viewed obliquely, and the pairs are not at the same level, a fact not shown in the drawing. In Fig. 4 there are six bodies, of which three show signs of bivalence. The others may be bivalent, but appear single because viewed from the end of the spindle. In late anaphases of the male-producing egg the chromosomes are always crowded. Five chromosomes can usually be recognized, but some specimens show six. From the existence of six pairs on the equatorial plate, I conclude that the number must be six even where only five can be recognized. The late anaphases which are favorable



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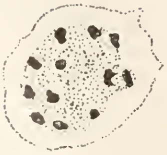

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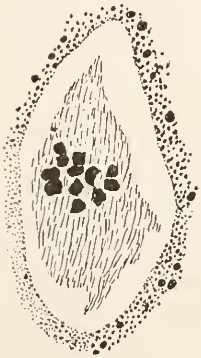

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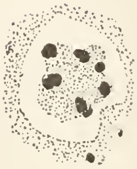

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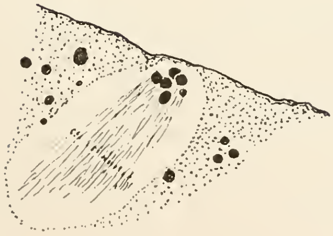

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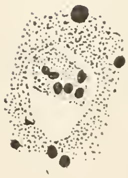

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Fig. 1. Maturation spindle of male-producing egg being formed within nucleus. A distinct nuclear membrane is still present. Only part of the spindle is in this section.

Fig. 2. Equatorial plate of maturation spindle of female-producing egg.

Fig. 3. Metaphase of first maturation spindle of egg of a male-producer. The spindle is viewed obliquely from the side, and the chromosomes are not all at the same depth in the specimen.

Fig. 4. Metaphase of the first maturation spindle in a male-producing egg. The view is nearly polar. Three of the chromatic bodies, set obliquely, give indications of being bivalent; the rest appear single.

Fig. 5. Portion of first maturation spindle, in late anaphase, of male-producing egg. The spindle is cut obliquely, and the outer end is here shown.

Fig. 6. Inner group of chromosomes, six in number, in late anaphase of first maturation division of male-producing egg.

enough to count include one or more groups at both outer (Fig. 5) and inner (Fig. 6) ends of the spindle, and the number is six in either case.

DISCUSSION.

From the best evidence I have been able to obtain, the number of chromosomes in *Hydatina senta* must be placed at 12. The number appears to be the same in male-producers and female-producers. This conclusion is less illuminating in some respects than might have been expected, but simplifies the probable chromosome behavior in certain parts of the cycle. The very sharply defined distinction between male-producers and female-producers, and the irrevocable determination of the nature of the egg at the time of maturation, would be regarded as "explained" if it could be shown that they rested on chromosome differences. If there are such differences, they are not differences of number. Whatever change occurs at maturation, to distinguish eggs that yield male-producers from those that yield female-producers, must, however, be quite as definite an event as the loss of a chromosome to the polar body.

With regard to the male phase of the life cycle, one may surmise the following consequences of the uniformity of chromosome number. The male probably develops with the haploid number of chromosomes, and in the maturation of his spermatozoa the reduction division is either abortive or suppressed. Or the male may at some stage double the number of chromosomes and maturation include a numerical reduction. In either case, the spermatozoa should contain six chromosomes. The sexual egg (identical with the male-producing egg described in this paper) presumably also has six chromosomes. At fertilization the number is restored to 12.

The status of the females hatching from fertilized eggs, which may be called the stem mothers, is a peculiar one. These stem mothers have been shown by hundreds of determinations to be always female-producers (Shull, '12). Whether, in the absence of differences in chromosome number, the feature which distinguishes a female-producer derived from a parthenogenetic egg from a male-producer is also the feature which makes a stem mother a female-producer can only be conjectured at present.

I am not prepared to advocate any view as to the method by which, in the absence of visible chromosome differences, a female-producer is distinguished from a male-producer. It has been suggested to me that there may be a difference in the chromatin, perhaps a quantitative difference, which does not involve the number of chromosomes. This possibility may be investigated in other ways. If there is a single fundamental distinction between the two kinds of females, any attempt to discover it must take account of the fact that the distinguishing feature of the female-producing parthenogenetic egg must also be an attribute of the fertilized egg.

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BIOLOGICAL BULLETIN

FERTILIZATION AND EGG-SECRETIONS.[1]

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I.

The newer work on fertilization is directly traceable to the investigations of Frank R. Lillie.[2] It was he who first drew our attention to egg-exudates: later, the loose ends of observation and experiment united, in his mind, into a scheme whose novelty has played the part of an intellectual ferment. If we can ask new questions and for that matter, answer them too, more precisely than we could in 1913, this is largely due to the catalytic effects of the fertilizin hypothesis.

From a large list of besetting problems—most of them only solved in part—I shall select for discussion merely those that have interested me particularly, and first among these is the question whether egg-secretions have anything whatever to do with fertilization.

Skeptics, now and again, have been caught coquetting with the inevitable difficulty that egg-secretions can be discovered and analyzed in only one kind of egg at a time. By insisting on this point they appear to attach comparatively little value to the distinctions between prevalence and importance. However, we are no longer terrorized by one who brandishes the single case. Today, exudates are known not only from the eggs of one echinoderm and one annelid, but from every one of the ten species of echinoderms that have been tested. Not merely this, but additional cases have been reported from annelids, molluscs and tunicates. To the list of previously recognized instances,

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[1] Read before the American Society of Zoölogists at a symposium on Fertilization, held in Chicago, December 30, 1920.

[2] An excellent summary of all the investigations in this field is to be found in Lillie's "Problems of Fertilization," University of Chicago Press, 1919.

I wish to add, parenthetically, another mollusc, the oyster, and two vertebrates, the fish, *Fundulus heteroclitus*, and the frog, *Rana pipiens*.

Though we may derive comfort from a distribution so varied, this in itself is no proof that the exudates are part and parcel of the mechanism of fertilization. Moreover the original method of demonstrating their importance is not free from suspicion. To wash eggs until all traces of their exudates have disappeared takes time; in the 18 to 36 hours necessary to accomplish this, the eggs themselves may undergo serious deterioration. Their failure therefore to develop if inseminated after washing cannot be attributed offhand to the removal of the exudates. Yet the original inference is correct[1] for the time required to remove the exudates may be cut in half by the use of running sea-water. By the use of charcoal[2] eggs may be freed from secretion completely in 3 to 4 hours; or, if the chorion is first removed, in 30 minutes. This falls well within the time limit within which unwashed eggs show no impairment of fertility. More convincing proof comes from experiments in which the fertility of eggs partially sterilized by the removal of exudate is measurably increased by the addition of freshly prepared secretion. Such an experiment with *Asterias* eggs was first carried out by Miss Woodward. Since then I have had an opportunity to verify her results on the eggs of *Echinarachnius parma*. These in a series of experiments were incompletely sterilized by the partial removal of the exudate. In one of the experiments the eggs were then divided into two lots; one of these was inseminated at once and several hours after fell into two groups—59 per cent. absolutely inactive ones and 41 per cent. in which development was going on. The latter group, however, was in turn divisible into cleavages patently anomalous and cleavages not distinguishable from the normal, approximately, in the ratio of 2 : 1. The second lot of eggs was treated with normal egg-secretion and inseminated in its presence. The results show a decrease in the inactive eggs to 50 per cent.—a decrease in the anomalous cleavages to 14 per cent. and an increase in normal divisions to 36 per cent.

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[1] There is one reservation; complete removal seems to have certain irreversible consequences. I have referred to the matter before. See this journal, Vol. XXVI., p. 395.

[2] The use of charcoal in this connection was suggested by Dr. G. H. A. Clowes. The suggestion was based on preliminary indications of the presence of enzymes and on the well-known efficacy of charcoal in removing these from solution. See Euler-Pope, "General Chemistry of Enzymes," p. 81.

II.

Our second question is, how do the exudates act? Naturally, we must formulate our explanation in terms of material entities. This, indeed, was done from the first, but the entity postulated was little more than a symbol for the observed effects.

These fall into two groups: the effects on spermatozoa and the effects on eggs. As Lillie first found, the spermatozoa are activated and exhibit a remarkable process of swarming and agglutination; the eggs, as I found, upon exposure to secretion develop spontaneously. Lillie attributed both phenomena to a single substance—the agglutinin of his earlier papers—the fertilizin of his later writings.

Lillie has introduced prevention as an aid to analysis, and has described two natural inhibitors. In the presence of one of these, the "anti-fertilizin" derived from the eggs themselves, spermatozoa do not swarm and agglutinate and eggs cannot be fertilized; in the presence of the other, contained in species-true blood, although the spermatozoa agglutinate, nevertheless the eggs fail to develop.

To account for these facts, Lillie made the following assumptions: The agglutinin has two bonds. One of these normally combines with a sperm-borne valence. The symptom of such union is the agglutination reaction. The other bond of the agglutinin unites with an egg-borne valence. The symptom of this union is the initiation of development. "Anti-fertilizin," prevents fertilization because it binds the spermophile group of the agglutinin and so renders the normal union with the sperm-valence impossible. Inasmuch as the inhibitor in the blood does not affect the agglutination reaction, this substance is assumed to occupy the ovophile bond of the agglutinin. Applying the picture-language of Ehrlich, Lillie called the agglutinin an "amboceptor," whose two bonds are satisfied in fertilization in the suggested manner.

The idea of attempting to isolate Lillie's amboceptor appeared foolhardy to many of my friends. As it was my own early attempts showed merely how it could not be done and what not to look for. The first ray of positive light came from the experiments of Richards and Miss Woodward with X-rays ('15). Their results suggested that the amboceptor might be an enzyme, and, as a matter of fact, by adapting a method previously employed by Robertson ('12) for the removal of oöcytase from ox blood, Miss Woodward succeeded in isolating a definite substance from the secretions of arbacia eggs ('18).

The substance in question was gotten in the form of a white powder easily soluble in sea water and in fresh. It did not cause the characteristic swarming and agglutination of the spermatozoa. It did however have decided virtues as a parthenogenetic agent. Moreover, it appeared to possess lipolytic properties. At any rate, oil droplets prepared from an ethereal extract of arbacia eggs, when exposed to a solution of the precipitate, decreased in diameter, in the course of two and a half hours, on the average, sixteen and seven tenths per cent. From this observation, Miss Woodward inferred that the fat was undergoing hydrolysis. Hence she called her precipitate, lipolysin.

Lipolysin is either not-agglutinin or it is denatured agglutinin. If the latter, then further search for an agglutinating substance in secretions from which lipolysin has been removed, should meet with little if any success. However a substance was isolated from just such remnants by saturation with ammonium sulfate, and the quantities so gotten appeared no smaller than those secured by the immediate saturation of the fresh exudates. The body precipitated in this fashion, when freed from salts, had no parthenogenetic properties. It did have marked powers as an agglutinant of sperm.

Whether Miss Woodward's further deductions and inferences will survive criticism matters not at all for the moment. Her results had the great virtue of preparing the ground for a different attack. In addition they made it possible to explain the effects of egg-secretions on spermatozoa and on eggs, in terms of two chemical individuals rather than in terms of one amboceptor with two side-chains.

This difference in the base from which interpretations must start, in the long run, may make small odds in the true inwardness of fertilization theory. I am not prepared to discuss the point. I am not even prepared to test the reasonableness of any particular answer pro or con for the conviction has overtaken me that I must practise birth-control in matters theoretical. But for one so minded, the one-body–two-body issue involves the whole matter of procedure. To me it is of great practical importance to determine, if possible, once for all, whether egg-secretions contain two substances or only one.

In his discussion of the question, Lillie[1] says: "This . . . does not explain why the sperm-agglutinating and the egg-activating properties of the egg-secretion always go together . . .; when the egg ceases to produce the sperm-agglutinating substance, it has lost its capacity to be activated. These two properties of the egg-secretion hang together normally; their separation under the conditions of chemical analysis may possibly denote a splitting of a single substance of the normal egg."

Unfortunately it would take us too far afield to discuss the essential question fully from the chemical side. Suffice it to say that during the last three years I have found other methods of isolation and in every case the agglutinating material and the egg-activating material are recovered as separate fractions. The amboceptor apparently has some pronounced physical weakness which causes it to break apart always at a point between the activating group and the agglutinating group. In fact this cleavage takes place so readily whenever one of the two groups combines with a precipitating agent that I have come to doubt whether the amboceptor can possibly hold together when its spermophile side-chain unites with the sperm-receptors.

However this may be, there is one bit of evidence which seems to me unassailable. Lillie proved that egg-secretions which have passed through Berkefeld filters do not cause the agglutinating reaction. The agglutinating material in this case, as Miss Sampson found last summer, and as I subsequently substantiated, can be recovered by washing the filter cone in sea-water. Whatever views one may hold regarding the physical-chemistry of filtration, that portion of the agglutinin which is recoverable in this manner is hardly the product of chemical breakdown suffered by the amboceptor molecule. For the ultra-conservative I may add that owing to the construction of the filter, a remnant of the secretion always fails to pass through. This remnant shows a higher agglutinating power than the original secretion. Moreover lest someone be led to suspect confusion, lipolysin can be isolated from the filtrates of such mechanical separations. Its presence in these is incidentally also revealed by activating effects on spermatozoa, a property the lipolysin shares with many other substances including preparations of pancreatic lipase.

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[1] "Problems of Fertilization," University of Chicago Press, p. 240.

III.

With our minds cleared on the one-body–two-body issue, we can ask and attempt to answer other specific questions. Among these we must include some further inquiries concerning the lipolysin. Is this substance really a lipolytic ferment? The evidence submitted so far is not conclusive because a decrease in the volume of oil drops might be the expression of changes in surface tension. But even if such shrinkage could be shown to result exclusively from hydrolysis, the question would still

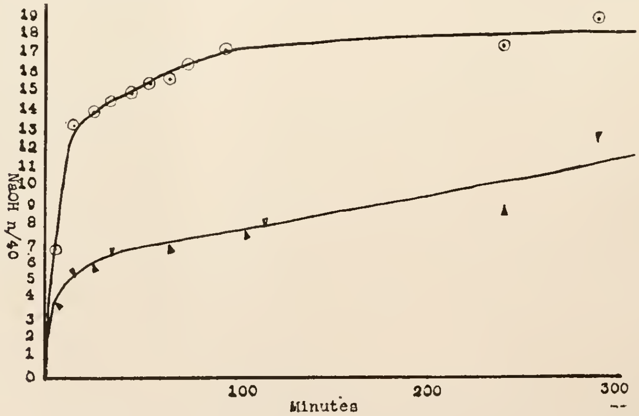

FIG. 1. Abscissa = minutes; ordinate = drops (1/100 c.c.) n/40 NaOH necessary to maintain system at PH 7. Both curves are the result of experimental averages and are slightly conventionalized. The lower one with experimental points at the apices of the triangles, represents the hydrolysis of ethyl butyrate in sea-water; the upper curve with experimental points in circles, represents the hydrolysis of ethyl butyrate in arbacia egg-secretion.

remain unsettled. An enzymatic effect is not demonstrated by hydrolysis, but by acceleration in the rate of hydrolysis.

Here again I cannot describe in detail the experiments which I have carried out during the last two years. However I am able to state very positively that the hydrolysis of neutral fats such as olive oil and whale oil as well as the cleavage of lower esters, such as ethyl butyrate are in fact accelerated not only by isolated lipolysin, but also by freshly prepared egg-secretions. The evidence, based on arbacia secretion, is given in the curves of Fig. 1. According to this lipolysin is correctly named for it is in fact a lipolytic ferment.

IV.

Can we come to similarly close quarters with the agglutinin? So far we have referred chiefly to the observable agglutination reaction. This, as Lillie found, is accompanied by other visible details. The agglutination of the spermatozoa is head-on. In *Nereis*, where the spermatozoa are larger than among the echinoderms, Lillie has described an actual swelling of the sperm-heads associated with a loss of normal refrangibility. He has suggested an increase of stickiness as the mechanism of agglutination.

The adhesiveness of cells unquestionably depends on surface properties and the effects of egg-exudates in general appear to be favorable to the view that agglutination is related to an incomplete superficial cytolysis of some sort. Aside from Lillie's evidence, which is susceptible to this interpretation, I have described a temporary agglutination of *Arenicola* larvæ accompanied by an outflow of pigment. Furthermore, isolated agglutinin has a marked effect on the surface of the egg. This quickly develops a clear zone immediately under the membrane—a result not only suggestive of membrane elevation but at the proper stage, quantitatively, perhaps the essence of this much debated process.

Richards and Miss Woodward, in their experiments with radiated egg-secretion, found evidence which suggests that very possibly the agglutinin also, is an enzyme. At any rate, radiation has opposite effects. If the secretion is radiated for two minutes, its efficiency as a sperm-agglutinant is increased, but it is decreased if the radiation is extended to fifteen minutes. Moreover, regardless of radiation, the efficiency of the agglutinin, like that of pepsin, varies with the square root of the concentration. The presence of carbon and nitrogen may be mentioned incidentally. However, if the agglutinin is an enzyme, it is not yet possible to suggest its taxonomic position among organic catalysts. Before this can be done, we must find the process which the agglutinin catalyzes.

V.

The last question I shall discuss is the relation of lipolysin and agglutinin to the specificities of fertilization.

We know that specificity is not absolute; it is often possible to fertilize the eggs of one species with the sperm of another, yet species-true spermatozoa always fertilize a much larger percentage of eggs.

The discovery that chemical entities traceable to the eggs themselves intervene in fertilization—however obscure such intervention may appear—has exposed the problem of specificity from a new angle. Lillie has discussed the matter very thoroughly in his book.

For me, the questions involved take the following form: Is it possible to substitute the lipolysin of one species for that of another? Is it possible to do the same thing with the agglutinins? With regard to the lipolysins, the following experiments may be cited: secretion was removed from the eggs of *Echinarachnius* until their fertility had been reduced to one half. Separate lots were then inseminated in the presence of arbacia, asterias, oyster, and species-true lipolysin—all in a concentration of ten milligrams to five c.c. of sea-water.

The results are given in Table I. in which are listed, in separate columns, the percentages of inactive eggs, those of the anomalous cleavages, and those of divisions indistinguishable from the normal. Apparently, it is not essential that the lipolysin be species-true. More dramatic than this, of course, is Miss Woodward's recently announced discovery that arbacia lipolysin is an excellent parthenogenetic agent for *Nereis* eggs.

TABLE I.

	Per Cent. Inactive Eggs.	Per Cent. Active Eggs.	
		Anomalous.	Normal.
Control	46	54	0
Arbacia lipolysin	41	57	2
Asterias lipolysin	28	66	6
Oyster lipolysin	26	62	12
Echinarachnius lipolysin	32	60	8

For the agglutinins, hybridization offers a good approach.

Just ('19) has made successful crosses between *Arbacia* and *Echinarachnius*. If now the agglutinins of these forms play a specific rôle in fertilization, one might expect that this could be rendered apparent in hybridization experiments. For this reason I treated the sex cells prior to insemination with species-true secretion and compared the results with crosses in which this treatment was not given. The experiments condensed in Table II. speak for themselves.

TABLE II.

Eggs.	Treated with Egg-Secretion.	Sperm.	Treated with Egg-Secretion.	Per Cent. Cleavage.
Arbacia	none	*Arbacia*	none	100
Arbacia	none	*Echinarachnius*	none	1
Arbacia	none	*Echinarachnius*	*Echinarachnius*	13
Arbacia	*Echinarachnius*	*Echinarachnius*	none	9
Echinarachnius	none	*Echinarachnius*	none	100
Echinarachnius	none	*Arbacia*	none	5
Echinarachnius	none	*Arbacia*	*Arbacia*	40
Echinarachnius	*Arbacia*	*Arbacia*	none	22

In these experiments I used none of the expedients so often employed to break down the incompatibilities that limit success in hybridization. Neither the eggs nor the spermatozoa were allowed to grow stale; no alkali was used; there were no repeated inseminations, nor were the spermatozoa added to the eggs in greater quantities than usual. With the sole exception of the treatment whose efficacy I wished to test, I followed strictly all the procedures of ordinary species-true insemination.

I think it must be admitted, not only that there is an effect, but that this effect applies primarily to the spermatozoa. Since now the lipolysins are apparently non-specific, I conclude, provisionally, that the agglutinating reaction involves specific elements in the sense that species-true agglutinin may have effects quantitatively and perhaps even qualitatively different from those of the heterologous agglutinins.

Whatever conclusions we may yet reach regarding the rôle of egg-secretions, we may legitimately at this time lay claim to success in certain directions and to some extent to forecast the future. Egg-secretions are not an isolated phenomenon; they have something vital to do with the initiation of development. Finally, the period of stumbling in the utter darkness is over, for our worst methods have reached some degree of reliability and some of them have yielded definite substances whose reaction-capacities mark off the zone within which we can hope to find understanding.

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HYDROGEN-ION CONCENTRATION OF PROTOZOAN CULTURES.

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The chemical changes taking place in ordinary hay infusions used for protozoan cultures have been described by various authors, among whom may be mentioned Peters[1] and Fine.[2] The points of interest discussed by these two authors are the changes occurring in the titratable acidity and alkalinity of such infusions. Changes in titratable acidity and alkalinity, however, as is well known, do not give a correct expression of the changes in concentration of hydrogen ions. Inasmuch as the hydrogen-ion concentration of the medium with which organisms are in contact is of great physiological importance, it was planned to study this factor throughout the life of series of protozoan cultures, and to see in how far such results agreed with those obtained by titration methods.

In the present study, therefore, determinations were made of both titratable acidity and alkalinity, as well as of hydrogen-ion concentration of series of cultures prepared in various ways.

The titratable acidity was obtained by titrating 5 c.c. of culture with 0.01 N NAOH, using phenolphthalein as indicator. The titratable alkalinity was obtained by titrating 5 c.c. of culture with 0.01 N H_2SO_4, using bromphenol blue as indicator. Hydrogen-ion determinations were made by colorimetric methods, using phosphate and acetate mixtures as standards, and phenolsulfonephthalein, brom-cresol purple and methyl red as indicators. Series of cultures were prepared in various ways and determinations carried out daily. In the case of hay infusion cultures with hay, the same amount of hay (approx. 25 gms.) was used and treated in the manner indicated in explanation of figures. The soil in soil cultures was obtained from the greenhouse and was that used by the gardener in ordinary greenhouse work. Total volume of cultures varied from 2 to 5 liters, depending on size of container used. When cultures were inoculated with organisms samples were taken from several cultures so that as many representative protozoan forms as possible were obtained. All cultures were kept under the usual laboratory conditions, in the same room, with the temperature as nearly constant as possible.

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[1] Peters, A. W., 1907, *Amer. Jour. Physiol.*, 17 and 18.

[2] Fine, M. S., 1912, *Jour. Exp. Zoöl.*, 12, p. 265.

The data obtained in such a study are most readily presented by curves, since it is relative and not absolute changes in which we are interested.

Peters[1] and Fine[2] found that the top and bottom of cultures varied in titratable acidity—at first, a high titratable acidity at the bottom, and later, as diffusion proceeded, a more or less uniform concentration was obtained and titratable acidity differences tended to disappear. A similar condition has been found by the author in titrations, but the differences in hydrogen-ion concentration are never great and, when occurring, last only for a very short time in the early life of the culture. In the following results, however, average conditions only will be given.

Figure 1 shows graphically the changes in Ph in a typical soil culture. These cultures, during the first few days, become slightly acid, followed by a less acid condition, and, finally, remain rather alkaline throughout the remainder of the experiment. Observation showed that organisms were most abundant during the early life of the culture, when the change from a slightly acid to a slightly alkaline reaction was taking place.

Figure 1 also shows the changes in Ph in hay infusion cultures prepared in slightly different ways. From these results it is quite evident that the presence of hay, either boiled or unboiled, tends to make a decided difference in the hydrogen-ion concentration of the culture. No attempt was made to keep the cultures sterile, and since it has been pointed out repeatedly that the bacteria are to a great extent, if not entirely, responsible for the acid production, these changes in Ph between cultures with and without hay are in all probability due to amounts of available food for bacterial organisms, as well as to the acid-yielding con-

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[1] Peters, A. W., 1907, *Amer. Jour. Physiol.*, 17 and 18.

[2] Fine, M. S., 1912, *Jour. Exp. Zoöl.*, 12, p. 265.

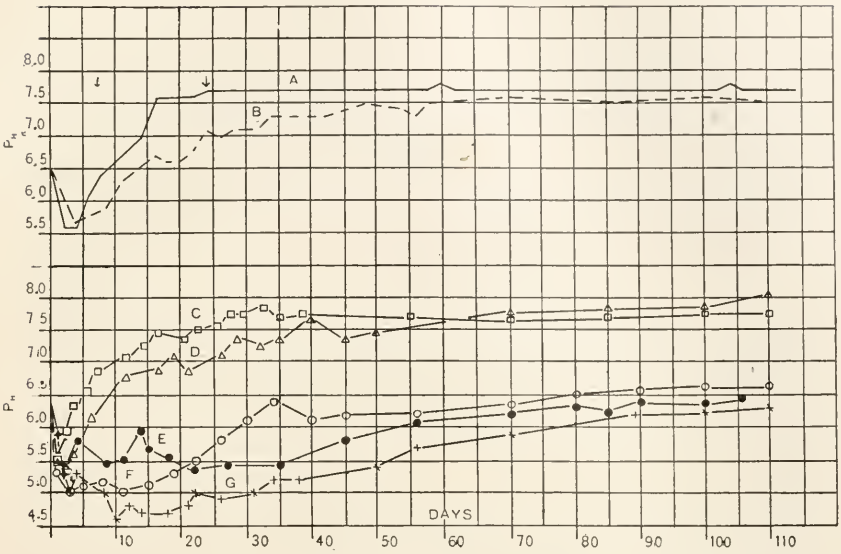

FIG. 1. *Top*—Curves show the Ph of soil and ordinary hay infusion cultures for a period of over 3 months.

A = soil culture, prepared with soil, hay infusion, tap water and inoculated with organisms.

B = culture prepared with hay infusion, tap water and inoculated with organisms.

Space between arrows indicates time when organisms appeared to be most abundant.

Bottom—Curves show the Ph of cultures with and without hay and also the Ph of inoculated and non-inoculated cultures.

C = culture prepared with hay infusion, tap water and inoculated with organisms.

D = culture prepared with filtered hay infusion and tap water and not inoculated.

E = culture prepared with hay, filtered hay infusion and tap water and not inoculated.

F = culture prepared with boiled hay, hay infusion, tap water and inoculated with organisms.

G = culture prepared with boiled hay, filtered hay infusion and tap water and not inoculated.

Volume of culture, 5 liters. Abscissas, time in days indicated. Ordinates represent Ph.

stituents of the hay. The differences in Ph between inoculated and non-inoculated cultures are probably due, either to a reduction of the bacteria by the protozoa, or possibly to various excretion products of the protozoa themselves.

It is also of interest to point out here that in the soil cultures there is a relatively short period of acidity followed by a longer

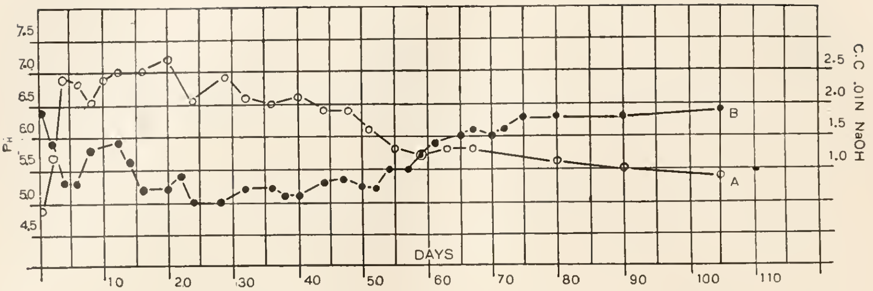

FIG. 2. Curves show the titratable acidity and Ph of an ordinary hay infusion culture, prepared with boiled hay, filtered hay infusion and tap water. *A* = titratable acidity. *B* = Ph. Abscissas time in days indicated. Ordinates at left represent Ph. Ordinates at right represent cubic centimeters 0.01 *N* NAOH required to neutralize 5 c.c. of culture.

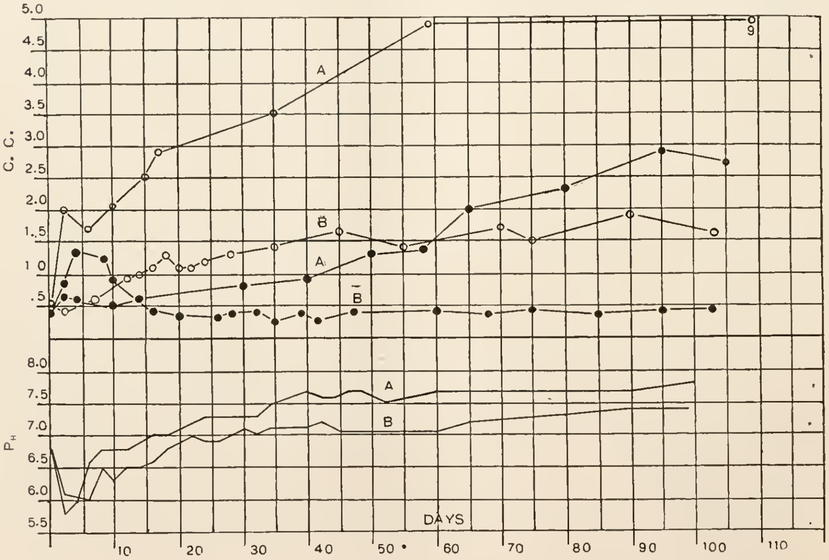

FIG. 3. Curves show the titratable acidity and alkalinity and the Ph of an ordinary hay infusion culture and of a hay infusion culture to which a piece of marble has been added. *A* = culture prepared with boiled hay, hay infusion, tap water, piece of marble and inoculated with organisms. *B* = culture prepared with hay infusion, tap water and inoculated with organisms. Volume of cultures, 5 liters.

Top—Curves show titratable acidity and alkalinity. O = alkalinity. ● = acidity. Abscissas, time in days indicated. Ordinates represent cubic centimeters 0.01 *N* NAOH and H_2SO_4 to neutralize 5 c.c. of culture.

Bottom—Curves show Ph of cultures. Abscissas, time in days indicated. Ordinates represent Ph.

one of alkalinity, while in hay cultures there is a period of acidity which lasts for a relatively long time in the life of the culture.

Figure 2 shows graphically the relation between the titratable acidity and hydrogen-ion concentration of a typical hay infusion culture. A somewhat more gradual and noticeable change is noted for titratable acidity than for hydrogen-ion concentration, and this seems to show that a marked change in titratable acidity is not necessarily accompanied by a correspondingly marked change in hydrogen-ion concentration.

Peters[1] and Fine[2] have shown that the acidity of hay infusions is due almost entirely to bacteria, to carbon dioxide, and acid-yielding hay constituents. This being the case, it is of interest to see how in an ordinary hay infusion a piece of marble will produce a fairly constant Ph.

Figure 3 shows graphically the changes in Ph and in titratable acidity and alkalinity in such a culture. These cultures gradually become alkaline in reaction, as indicated by Ph, but still have an appreciable titratable acidity. Cultures prepared in such a manner ought to be useful for keeping organisms in media of almost constant Ph.

The above results on titratable acidity and alkalinity are quite in agreement with those reported by Peters[1] and Fine,[2] and in addition show the relative changes in hydrogen-ion concentration during the life of hay infusion and soil cultures. The sequence of organisms and changes in hydrogen-ion concentration have not been particularly studied, but it is not improbable that some relation between the two might be shown to exist.

[1] Peters, A. W., 1907, *Amer. Jour. Physiol.*, 17 and 18.

[2] Fine, M. S., 1912, *Jour. Exp. Zoöl.*, 12, p. 265.

THE AXIAL GRADIENTS IN HYDROZOA.

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IV. AXIAL GRADATIONS IN RATE AND AMOUNT OF REDUCTION OF POTASSIUM PERMANGANATE IN VARIOUS HYDROIDS AND MEDUSÆ.

In an earlier paper (Child, '19*a*) attention was called to a method of rendering directly visible the physiological axial gradients in many of the simpler organisms and earlier developmental stages through the differences in rate and in total amount of reduction of potassium permanganate by the protoplasm of different regions and levels. Since the reduced permanganate (MnO_2 or other oxides) colors protoplasm brown to opaque black according to amount of deposit, differences in rate or amount of reduction in different regions appear as differences in depth of coloration or in degree of opacity.

In the paper referred to, the results of work with permanganate on various animal and plant species are briefly described and it is pointed out that the gradients in rate and amount of permanganate reduction correspond to the physiological gradients demonstrated by means of the susceptibility method and various other methods.

MATERIAL AND METHOD.

The present paper records observations made on the reduction gradients, as they may for convenience be called, of various species of hydroids and medusæ. The work was done at the Puget Sound Biological Station chiefly during the summers of 1918 and 1919 and I am indebted to the director for the privileges of the station. The data concerning the hydroids were obtained chiefly from four species, *Bougainvillea mertensi*, *Gonothyræa clarki*, *Obelia borealis* and *O. longissima:* but observations on rate of reduction were also made on several other campanularian species. Young hydroids were grown in the laboratory from the eggs of *Gonothyræa* and the hydromedusa *Phialidium gregarium* and the physiological gradients have been followed through all developmental stages by various methods, but consideration of the embryological data is postponed to a later paper. Among the hydromedusæ four species, *Phialidium gregarium*, *Mitrocoma discoides*, *Æquorea cærulescens* and *Sarsia rosaria* constituted the chief material for the work with permanganate, but observations were made as opportunity offered on several other forms, *Stomatoca*, *Obelia* and several undetermined species.

The experimental procedure consists merely in immersing the species to be examined in a solution of $KMnO_4$ in sea water: For observations on the color gradients resulting from differences in rate of reduction concentrations ranging from $m/1000$ $KMnO_4$ down to $m/10,000$ or even less have been used in most cases. Higher concentrations than $m/1000$ may of course be used, but in such concentrations the color of the solution is so deep that the animals must be removed to water for observation. Moreover, the differences in rate of reduction and staining are more distinct in the lower than in the higher concentrations because the latter kill all parts almost at once in spite of the differences in physiological condition. Differences and gradients in total amount of reduction are determined by permitting the reduction to proceed to completion in excess of permanganate. Under these conditions most organisms become opaque black and no differences in depth of color can be seen, but in certain small organisms, embryonic stages, or regions of body, in which the thickness of protoplasm or cell mass is not too great, the color gradient resulting from differences in amount of MnO_2 deposited is directly visible. Many other forms which are opaque black in water may be made more or less translucent by hardening and gradual dehydration in alcohol and clearing in an oil, and if desired mounting in balsam. The axial gradients in blastulæ and other early embryonic stages of various species, as well as in the adults of various small forms, can be seen with great distinctness in such preparations. Larger organisms or regions which reduce relatively large amounts of permanganate may remain opaque even after clearing. In fact, the method is chiefly of value for small, more or less translucent organisms, but for these it gives very definite, uniform and beautiful results.

I have made no attempt to determine at all exactly the length of time necessary for completion of the reaction in given concentrations but have merely made certain that length of time was sufficient for the purpose. Usually preparations of this sort remained 24–48 hours in a solution of *m*/1000 or higher. My observations indicate, however, that in small organisms, such as hydroids, and in embryonic stages the reaction is in most cases complete in much shorter time, *e.g.*, 2–4 hours in *m*/1000.

All concentrations of $KMnO_4$ high enough to give any appreciable deposit of the oxide on or in the protoplasm are highly toxic. Even in *m*/20,000 ciliary activity ceases within a short time, ranging from a few seconds to about one minute in forms examined and traces of the color begin to appear on the external surfaces within two or three minutes. Except in those concentrations in which cytolysis and disintegration take place it is impossible to determine exactly when death occurs. Undoubtedly it occurs before reduction and staining proceed very far, for alteration in aggregate condition, apparently a coagulation, can often be observed in the cells in early stages of coloration.

Certain precautions necessary in the use of the permanganate may be noted. Certain low concentrations produce cytolysis and disintegration in many, perhaps in all, organisms. In such cases susceptibility gradients corresponding to those observed with other agents and to the reduction gradients appear. In concentrations producing disintegration the disintegrated mass may show more or less distinct gradients in rate or amount of reduction, provided it holds together, but commonly cytolysis is followed by loss of continuity and by distribution of the cell substance through the water. In higher concentrations the reduction gradients appear without disintegration.

In order to avoid irregularities in staining it is necessary to agitate the solution frequently or to move the organisms about in it. Reduction of the permanganate at the surface of the organism often occurs much more rapidly than its diffusion, and a zone of low concentration of permanganate may appear about the organism, particularly about its more rapidly reducing regions. Parts in contact with the glass or lying near other reducing parts soon show a retardation in staining in consequence of decrease in concentration of permanganate in the region about them. For these reasons the volume of solution used should be large as compared with the volume of protoplasm and uniformity of concentration at all points of the protoplasmic surfaces should be maintained by continuous or frequent agitation.

One difficulty exists in the technique of permanent preparation of permanganate material either for whole mounts or for sections. All clearing agents used thus far remove more or less rapidly the black or brown deposit in the protoplasm, the MnO_2 being apparently soluble in or reacting in some way with the oils. This disappearance of color also occurs in Canada balsam. In the samples of clove oil used the disappearance of the color is rapid, *e.g.*, hydranths may fade from opaque black to light uniform yellow in two or three days. In cedar oil the fading occurs much more slowly and may be a matter of weeks. In the course of a study of permanganate preparations of larval stages of echinoderms Mr. A. E. Galigher has found that with rapid preparation sections could be obtained without appreciable loss of color, but that in balsam the color gradually disappeared. Small organisms, larval stages, etc., can be made sufficiently translucent by dehydration and clearing so that the differences in total amount of reduction are clearly visible and persist at least for a day or two, or with some clearing agents much longer.

It is scarcely necessary to point out that differences in thickness of the layer of protoplasm through which the light is transmitted may appear as differences in depth of staining. A hydroid stem of larger diameter, for example, may appear to be more deeply colored than a stem of smaller diameter and in such cases it is often quite impossible to determine whether the difference is real or apparent. Such difficulties arise chiefly in connection with preparations for total amount of reduction rather than with those for rate of reduction, for in the latter reduction and coloration occur first on the surface and progress inward, and the differences in rate of staining can usually be seen in early stages on the external surface of the protoplasm quite independently of its thickness.

Thus far an exact quantitative record of the differences in rate of staining has not been attempted because of the difficulties involved. It is difficult to determine exactly the moment when the staining begins in a certain region and although the regional differences in depth of color are very marked, even in forms as small as the hydroid planula or sea urchin blastula and gastrula, any adequate measure of this difference is not readily obtained. Photomicrographs can undoubtedly be made of stages of staining or of the cleared preparations, but in any case are little better than figures, and graphic methods are much less readily applied than in case of disintegration experiments (Child, '15, Chaps. III.–VII.). In the present paper description is supplemented by a few diagrammatic figures in which the regional differences are indicated at some stage of coloration by degrees of shading.

Reduction Gradients in Hydroid Colonies.

Before describing the details it may be said that in general both the rate and, so far as it could be determined, the total amount of reduction, decrease from the axial regions basipetally. This holds not only for the single hydranths, hydranth buds, growing tips, medusa buds and stolons, but for the colony as a whole. The differences at different levels are least in the stems. The perisarc is of no very great significance as an obstacle to the passage of permanganate to the tissues within it. In the more apical regions parts inclosed in perisarc begin to stain almost or quite as quickly as naked parts, and even the thicker perisarc of the more basal regions of the stem retards the staining only slightly. The thickness of the perisarc, therefore, is certainly not responsible for the differences in rate of staining of different levels. The deposition of the oxide begins on the external surface of the protoplasm and a distinct regional gradient in staining is in many cases visible on the surface before any trace of reduction appears below the surface.

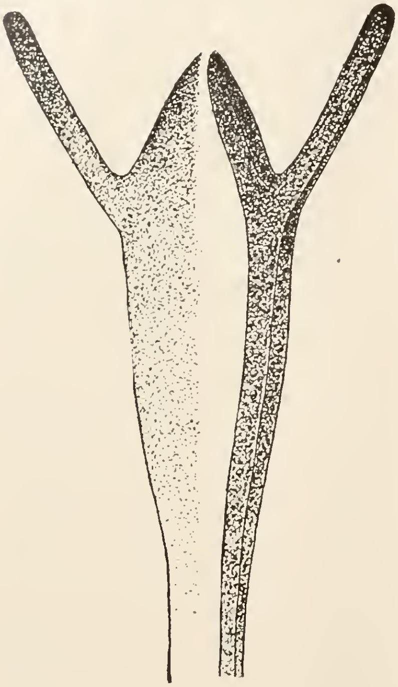
The Hydranth Gradients.—Reduction and coloration occur first at the tips of the tentacles and within a few moments each tentacle shows a distinct color gradient ranging from brown at the tip to light yellow in the basal region. Color begins to appear at the tip of the manubrium shortly after it appears on the tentacles, never, so far as my observations go, at the same time, and progresses basipetally on the manubrium, the basal portions of the hydranth body being the last to show color. The color appears first on the external surface and it is not infrequently possible to see the formation of precipitate on the external surface of the cells. Such precipitate must result from reduction of permanganate on the external surface of the protoplasm and the reduction and coloration progress from the surface inward. The permanganate does not penetrate the living cell and kill after it attains a certain concentration in the interior but it reacts with the protoplasm and undoubtedly kills it as it comes into contact with it.

The color deepens rapidly and within fifteen minutes to an hour in concentrations ranging from $m/2000$ to $m/7500$ the regions where coloration first appeared are deep brown or opaque black and from these a color gradation to yellow in the basal regions appears.

After two to three hours the whole hydranth is usually opaque black in water. Teasing shows that at this time reduction has occurred throughout the ectoderm and that the entoderm is more or less deeply colored, and sometimes gradual differences in rate of entodermal staining can be seen, but the existence of entodermal gradients can be determined with certainty only in dehydrated and cleared material. After reduction is completed all hydranths are opaque black in transmitted light, and even after dehydration and clearing the bodies of the larger hydranths are opaque throughout, but the gradient is distinct in all tentacles and in at least the smaller hydranths.

The diagrammatic Fig. 1 indicates the color gradients in the tubularian *Bougainvillea*. The lighter side of the figure shows the gradient in rate of staining in single tentacle and the hydranth body, as seen from the surface after fifteen minutes to one hour in $KMnO_4$ according to concentration. The right side of the figure represents an optical section of tentacle and body wall after complete reduction, dehydration and clearing. The shading is intended to give some idea of the differences in depth of staining at different levels. After total reduction and clearing all parts are very deeply colored, in fact large hydranths are often still completely opaque. The gradients are similar in the campanularian hydranth.

The Gradient in Hydranth Buds.—In *Bougainvillea* the buds show very distinct apico-basal gradients in rate of reduction from early stages on, and in material dehydrated and cleared after complete reduction the basipetal gradient appears in both ectoderm and entoderm. Figure 2 shows a somewhat advanced bud, the left side in surface view indicating the gradient in rate of reduction, the right side in optical section indicating the gradient in amount of reduction in cleared material.



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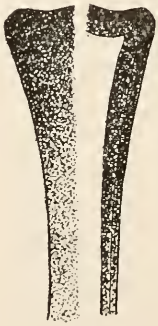

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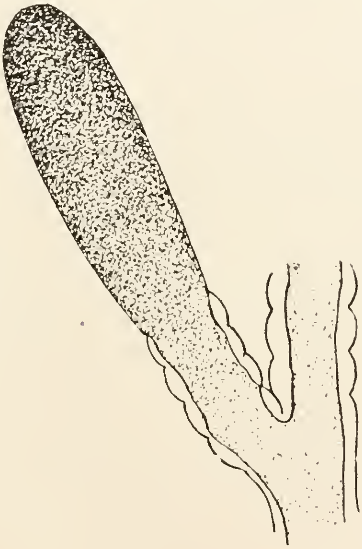
In *Obelia*, *Gonothyræa* and other campanularians the axis of the colony is, as I have pointed out, sympodial and the bud which originated as a lateral bud below the terminal hydranth or the terminal bud undergoes considerable growth in length as a continuation of the main axis of the colony and may even give rise to a new bud or branch before a hydranth develops at its tip (see Child, '19*b*, Fig. 10). These terminal regions of the campanularian colony and of its branches are regions of relatively rapid growth, each one representing temporarily a growing tip, and reduce $KMnO_4$ rapidly and in large amount. In spite of the fact that it is covered with thin perisarc, the coenosarc at the apical end of such a growing tip begins to stain earlier than any other part of the colony except the tentacles and in some cases the hypostome regions of hydranths. These growing tips and the hydranth buds which develop at their apical ends always show distinct basipetal gradients in rate of reduction, as indicated in Figs. 3 and 4. In preparations cleared after reduction is complete these parts in vigorous, rapidly growing colonies are usually stained so deeply that they remain opaque even after clearing, but these preparations show a much greater amount of reduction in these terminal regions than in the stems.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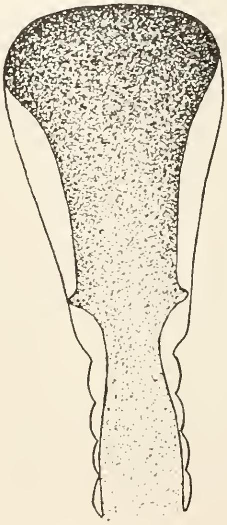
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The Gradients in Stems.—All parts of the stem are of course enclosed in perisarc which increases in thickness basipetally. Reduction of permanganate does occur to a slight extent in the perisarc; in the thin perisarc of the more apical regions the resulting color is scarcely appreciable, but the thicker perisarc of the more basal regions, which is normally yellowish or brownish in most forms, appears more deeply colored in the permanganate, probably because of its greater thickness. In no case observed, however, is reduction in the perisarc sufficient to obscure the progress of coloration in the cœnosarc.

The permanganate penetrates the perisarc rather rapidly. The apical regions enclosed in very thin perisarc begin to stain almost as soon as the naked parts. Penetration of the thicker perisarc of the more basal regions occurs somewhat more slowly, though even in these regions the perisarc is far from being impermeable. Because of the presence of the perisarc and its different thickness at different levels, difference in rate of reduction and staining of intact stems cannot be regarded as a safe criterion of the differences in physiological condition. This difficulty, however, is easily avoided by using pieces of stems from different levels with fresh transverse cut surfaces. Pieces from different levels may be placed side by side in permanganate and the rate of coloration of the exposed distal or proximal ends directly compared. In this way it is readily determined that a gradient in rate of reaction is present in the stems. This gradient is much less steep than that of hydranths and growing tips, for reduction occurs relatively slowly in all stem regions, but with differences in level of 10–15 mm. or more it is appreciable, and between stem levels near the apical and those near the basal end of colonies several centimeters in length the difference is marked. In such cases the more apical level usually becomes distinctly yellow or even brownish before the more basal level is appreciably colored.

In preparations of whole colonies or of the longer branches, cleared after completion of reduction, the stems may remain opaque throughout if the colony is well fed and the cœnosarc thick, but in most cases a distinct basipetal decrease in opacity appears. In order to make certain that such differences are not merely apparent and due to differences in transverse diameter of the perisarc, it is desirable to compare regions from the different levels in which the diameter of the cœnosarc is approximately the same. Repeated observations of this sort leave no doubt that real axial differences in the total amount, as well as in the rate of reduction exist in stems and branches.

The Stolon Gradient.—Many hydroid species give rise under slightly depressing conditions to stolon outgrowths, not merely from basal or even from apical cut ends, but by transformation into stolons of hydranth buds, in campanularians, of growing tips or even apical ends or stems after degeneration of hydranths. Experimental data concerning such transformations will be presented in another paper. The stolon is readily distinguishable from the stem by indefinite direction of growth, by attachment to the substratum or other solid objects and in the campanularians by absence of the annulations characteristic of stems. The stolon, like other parts of the colony, shows a gradient both in rate and amount of reduction, decreasing from the tip. This gradient is less steep than that of the hydranth or the growing tip, but steeper than that of stems. Reduction of permanganate in the growing stolon is more rapid than in the stem, but less rapid than in hydranth buds and growing tips.

The Gradient of the Colony as a Whole.—The reduction gradient appears, not merely in the individual zooids and parts of the hydroid colony but there is a general gradient characteristic of the whole colony. The gradient of stem and branches already described above is of course a part of this general gradient, but this gradient also appears, like the susceptibility gradient (Child, '19*b*) in the differences between the hydranths and growing tips of different regions.

The *Bougainvillea* colony is monopodial like *Pennaria* (Child, '19*b*) and the primary hydranth of the colony or branch remains permanently the apical hydranth. Budding occurs subapically at a certain distance below the apical hydranth, and the new axes developing from the buds become lateral branches of the colony or branch each bearing its primary apical hydranth. In any complex colonial axis, the lateral branches grow less rapidly than the main axis and the physiological relations between the different axes are apparently similar to those existing in plants of similar growth-form, *i.e.*, the chief apical region inhibits in some way and to some extent the lateral branches. It has been shown (Child, '19*b*) that these complex axes possess a general susceptibility gradient, the apical hydranth of the main axis being in general more susceptible than the apical hydranths of lateral branches and the apical hydranths of the more apical lateral branches, more susceptible than those of more basal branches. In *Bougainvillea* this general gradient is less distinct than in some of the campanularians with more definite growth-form.

In *Obelia* and *Gonothyræa* a similar general gradient appears in the whole colony and in the complex axial systems represented by its branches. In these forms, however, the growth form is sympodial (Child, '19*b*) and instead of a permanent apical hydranth each growing tip and the hydranth bud developing from it is temporarily apical, but is displaced by the next bud. The general colony gradient appears in the rate of reduction of the different growing tips and hydranth buds of an axial complex, the most apical showing in general the most rapid reduction.

In vigorous, rapidly growing colonies of *Obelia borealis* 5 cm. or more in length, taken from piles and floats, the general gradient is usually clearly visible to the naked eye by reflected light after reduction is completed or has progressed so far that the tissues are opaque in water. On removal to water and washing the depth of brown color of the tissues of such a colony, as seen by reflected light, decreases in general from apex to base in the colony as a whole and in each axial complex. The most apical growing tips and buds are very deep brown, almost black, the growing tips and buds of more basal regions distinctly lighter in color and even in the developed hydranths similar differences appear to some extent. The color gradient in the stem also appears in spite of the fact that the thicker perisarc of the more basal regions is more deeply stained by permanganate than that of more apical regions.

It is perhaps necessary to emphasize the point that these general gradients are most distinct in vigorous, growing colonies. The observations recorded above were made on colonies which were known to have developed within three to four weeks preceding the observations and in which medusa buds or gonozooids had begun to appear only in the basal regions. I have pointed out elsewhere (Child, '19*b*) that medusa-bud development normally begins in colonies which are relatively old physiologically and in the most basal regions of the colony, *i.e.*, in the region of least susceptibility, and as the data of the present paper show, of lowest rate of reduction of permanganate. Later, as the colony grows progressively older, medusa-buds or gonozooids appear farther apically, until finally they may develop even from the apical regions.

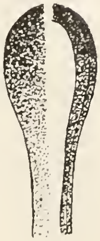
The old colonies with medusa-buds or gonozooids in or near the apical regions may show little or no indication of a general colony gradient. Often in such cases the original hydranths of the most basal levels have disappeared, and in some cases new young hydranths may be developing. Colonies in this stage often show a higher susceptibility and higher rate of reduction in the newly developed, physiologically younger hydranths or growing tips of the more basal regions than in those of the more apical regions, which represent the original generation. Later, when these also have been replaced by a new generation the general colony gradient may once more become like that of the young growing colony.

In short, the gradient is not a fixed unchangeable condition which can be demonstrated in any hydroid colony. It is a feature of the normal developmental and functional relations of parts, but may be altered, obliterated or even reversed by environmental or physiological conditions. The gradients in the single hydranths and growing tips are more persistent. So far as my observations go, their disappearance is followed by the disappearance through death in the case of more highly specialized, or resorption in the case of less high specialized parts. Similarly, in the colony as a whole the disappearance of the general gradient is associated with the disappearance, at least temporarily, of the original order and relation characteristic of the colony. Old hydranths or axial complexes may die or be resorbed and new ones may develop, but my observations indicate that wherever new development of a hydranth or an orderly axial complex occurs the characteristic gradients are found to be present and after a period of resorption, redifferentiation and rejuvenescence following the stage of medusa-bud formation the general colony gradient may reappear simply as a physiological consequence of the order in which development, senescence, death or resorption, and new development occur in different parts of the colony. In other words, the new colony gradient in such cases is a physiological consequence of the existence of the original gradient. Of course, in some cases, environmental factors may play a part in altering the original relations and so in modifying the form and order of the colony.

It is of interest to note that Gast and Godlewski ('03) studying the form regulation of *Pennaria cavolinii* found a general colonial gradient in rate of regeneration of hydranths. After removal of the hydranths from a colony or axial complex, development of new hydranths decreased in rate basipetally in the colony as a whole and in each axial complex. Later degeneration of the regenerated hydranths began in the most basal regions and proceeded a greater or less distance apically, but the most apical hydranths persisted, grew and budded at the expense of these other more basal parts. Both of these gradients, the regeneration gradient and the degeneration gradient, indicate that the fundamental physiological activity of the protoplasm is greater apically and decreases basipetally in the colony as a whole and in each axial complex. In short, these regulatory phenomena constitute still another line of evidence for the existence of the physiological gradients.

And finally, I am permitted to state from unpublished work that Dr. A. W. Bellamy and Dr. L. H. Hyman, working independently, have found gradients in electric potential in hydroid colonies of various species. These electrical gradients correspond to the gradients in susceptibility, reduction of permanganate and rate of regulation. In vigorous, growing colonies the apical region of the colony is galvanometrically negative to all other levels, the apical region of each axial complex is negative to all other parts of that complex, and the apical regions of the more apical branches are negative to the apical regions of the more basal branches.

The Gradient of the Medusa Bud.—In the naked medusa buds of *Bougainvillea* and other tubularians the gradients in rate and amount of reduction of $KMnO_4$ appear very distinctly. The free apical end of the bud always shows the highest rate and greatest amount of reduction, as indicated in Fig. 5, a young bud of *Bougainvillea.* The medusa buds of the campanularians constitute less favorable material because the crowding along the blastostyle and the presence of the gonotheca render it impossible to insure uniform concentration of permanganate over all parts of the surface. In general, however, it is perfectly clear that the apical regions of these buds reduce permanganate more rapidly and in greater amount than other levels.



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In *Gonothyrœa* the rudimentary medusæ or gonophores in the later stages of development come to lie outside the gonotheca, though still attached to the blastostyle, and so are directly exposed to permanganate solutions. The rate of reduction is highest in the small tentacles of these gonophores and decreases basipetally over the outer surface of the body. The subumbrellar cavity of the gonophore is at first closed and never widely open to the exterior, so that it is not possible to determine with certainty the relative rate of reduction in this region, though it is probably much the same as in other medusæ. In the old gonophores after extrusion of the planulæ the reduction gradient may be almost or entirely absent.

Reduction Gradients in Free-living Hydromedusæ.

The simple experiment of placing medusæ in a permanganate solution and agitating sufficiently to provide for uniform distribution of the permanganate was performed repeatedly during three summers and always with essentially the same result. In all forms examined reduction occurs most rapidly in the marginal tentacles and the oral lobes or tentacles, when such are present. Each tentacle or lobe shows a gradient in rate of reduction, decreasing basipetally. Elongated manubria show similar gradients in rate of reduction. Manubrium and margin are regions of more rapid reduction than other subumbrellar regions, but the subumbrellar ectoderm in general reduces more rapidly than the exumbrellar ectoderm. Dehydration and clearing is not necessary in the medusa species used because the cellular tissues are mostly in such thin layers that they do not become opaque black. Nevertheless, the final difference in depth of color between exumbrella and subumbrella cannot be regarded as a fair criterion of differences in total amount of reduction because the thickness of cellular tissue is much greater in the subumbrella than in the exumbrella. On this account I am inclined to regard the differences in rate of reduction as more significant than the final differences in depth of color. The jelly reduces little permanganate and reduction is very slow.

Data on susceptibility of medusæ have not previously been recorded, but numerous experiments have been performed, chiefly on *Æquorea*, but in part on other species, with various concentrations of several agents, viz., KNC, $m/100$–$m/500$, HCl, $m/200$–$m/800$ and KOH, $m/200$–$m/600$. The susceptibility gradients in all these agents are essentially the same as the reduction gradients. A few experiments with neutral red and methylene blue and various other agents on the smaller forms gave similar results. It may be said then that here, as in the hydroids, the susceptibility gradients and the reduction gradients correspond.

It is of interest to note that the higher susceptibility and more rapid reduction of the subumbrellar as compared with the exumbrellar regions is in accord with McClendon's data on oxygen consumption in *Cassiopea*. He found that the subumbrella consumed much more oxygen per unit of weight than the exumbrella plus the greater part of the mesoglœa (McClendon, '17). Dr. Bellamy and Dr. Hyman have found differences in electric potential corresponding to the differences in susceptibility and rate of reduction, not only as regards subumbrella and exumbrella, but also as regards marginal and oral, as compared with other subumbrellar regions. The regions of highest galvanometric negativity correspond in all cases to the regions of highest susceptibility and rate of reduction.

Reduction after Killing.

The gradients in rate and amount of reduction of permanganate appear when the living animals are brought into permanganate, but when they are first killed by some other means and then brought into permanganate the gradients are either entirely absent, or only the merest traces of them remain. Various means of killing have been used in these experiments, among them $HgCl_2$, $HgCl_2$ with 5 per cent. acetic acid, HNO_3, HCl, strong alcohol, formalin, hot sea water. If the animals are brought into permanganate within a few moments after the killing agent is applied, with only a brief washing in sea water, if necessary to remove excess of the killing agent, slight gradients may still appear. So far as my observations go, however, this is not the case if the animals are subjected after killing to the usual treatment of histological material. If, for example, they are passed through the alcohols up to 70–80 per cent. and kept in this percentage for a day or two, or if they are killed and kept in formalin and then washed in water before being placed in permanganate, the rate of reduction in different parts is uniform and the only differences in depth of color, so far as could be determined, are the apparent differences resulting from differences in thickness of the layers through which the light passes. Moreover, in these killed animals the total amount of reduction is much less than when the living animals are brought into permanganate. Potassium permanganate, even in high dilutions, is highly toxic and when the living animals are placed in it they are of course killed by it, but it is evident from the facts cited that the differences in physiological condition in different regions play a part in determining the rate and amount of reduction. In the dead animals these differences are no longer present, although some factor or factors concerned in them may persist for a time after the killing agent is applied. Whatever the nature of the differences in reaction to permanganate between living and dead protoplasm, it is evident that the gradients in rate and amount of reduction are associated with and dependent upon the living condition and disappear, either with, or soon after, death.

CONCLUSION.

Since $KMnO_4$ is a powerful oxidizing agent, the inference is justified, as I have pointed out elsewhere (Child, '19*a*), that the rate and amount of reduction of permanganate by living protoplasm is in some way and to some degree related to oxidative activity in the protoplasm. This inference is apparently supported by the marked decrease in reducing capacity of the protoplasm with death and the disappearance with, or soon after death, of the regional differences or gradients in rate and amount of reduction. Moreover, the reduction gradients are essentially the same as the susceptibility gradients and many different lines of evidence indicate that the latter are, at least to some extent, associated with and dependent upon differences in rate of oxidation (Child, '20). The results obtained with permanganate are then in complete agreement, as regards the existence of physiological gradients, with those obtained by other methods and constitute another line of evidence in support of the general conception.

The question of the rôle of permeability in relation to the reduction gradients must be raised. It has been repeatedly pointed out (Child and Hyman, '19, Child, '20) that gradients in permeability are in many, if not in all cases, characteristic features of the physiological gradients, but many facts show clearly enough that the fundamental metabolic activity of the protoplasm is also concerned in these gradients. We are forced to conclude, either that the gradients are not simply permeability gradients, or else that permeability and the fundamental metabolism of the protoplasm are very intimately associated and more or less interdependent. As regards the reduction gradients, it may seem at first glance that the differences in rate of reduction are merely the result of differences in permeability to permanganate. This is perhaps to some extent the case, but observation indicates that these differences appear first upon the external surface of the protoplasm. It is not necessary that the permanganate shall penetrate to the interior of the cell before the reaction begins. Moreover, the protoplasm in which the reaction occurs is undoubtedly killed very rapidly, as is indicated by the almost instantaneous cessation of ciliary and flagellar activity, even in very low concentrations of permanganate. It is certain that the permanganate does not penetrate the plasma membrane or any other limiting surface to any appreciable extent while that surface is living, but rather kills or begins killing as it comes into contact with the surfaces. My observations indicate, however, that the differences in rate of reduction disappear after death. If this is true, permeability in any physiological sense, *i.e.*, permeability of living protoplasmic limiting surfaces, cannot be the chief factor in determining the gradients in rate of reduction, for any differences in such permeability must disappear very soon on contact with the solution. If the particular limiting surfaces persist as limiting surfaces after the action of permanganate, and it is by no means certain to what extent they do persist, their permeability is no longer the physiological permeability of the living surface, but simply that of a dead surface. In short, after a given limiting surface, whether external or internal, has been killed by permanganate, the further passage of that surface by permanganate is not determined by its physiological permeability. If this argument is correct, the differences in rate of reduction of permanganate determined by differences in physiological permeability should be slight, since permanganate is highly toxic, but, as a matter of fact, the differences are very great when living animals are brought into the solutions and absent, or almost absent when dead animals are used.

Moreover, the deposition of MnO_2 or other oxides in the protoplasm is the result of a chemical reaction between the permanganate and protoplasmic constituents and therefore depends on other factors than the mere entrance of the agent. There is every reason to believe that the chemical and particularly the oxidative activity of the protoplasm is a factor in determining the velocity of the reaction, even though death occurs rapidly. Exactly what changes constitute death in permanganate, or for that matter in any other agent, it is impossible to say, but it is evident that the beginning of death does not stop the reduction of permanganate, and even after death is complete the reaction occurs to some extent. The slight regional differences in rate of reduction which sometimes persist for a time after death is apparently complete may perhaps mean that certain death changes, *e.g.*, inactivation of oxidizing enzymes, stabilization of oxidizable substance, are not yet complete, but at most these differences are mere vestiges of the original differences.

And finally, the regional differences in the total amount of reduction cannot be due to differences in the permeability of limiting surfaces. In the light of all the facts concerning the physiological gradients, the only conclusion at present justifiable seems to be that they are associated with and dependent upon differences in the chemical, and particularly the oxidative activity of the protoplasm concerned, though it is, of course, granted that various factors are concerned in these chemical differences. The reduction gradients appear only when living protoplasm reacts with permanganate, or as mere vestiges in protoplasm very recently killed. The regions of greatest amount of reduction are the regions of greatest physiological activity, as indicated by growth, development, susceptibility, electro-negativity, and in those forms for which it has been determined, of respiratory activity. I believe we are justified in concluding that, when used with proper precaution and in connection with other methods, the rate and amount of reduction of potassium permanganate by organisms which are alive at the beginning of the reaction serves as an indicator of the fundamental physiological condition of the protoplasm. This conclusion holds, not only for hydroids, but for all other forms examined (Child, '19*a*).

SUMMARY.

Hydroids and hydromedusæ placed in dilute solutions of potassium permanganate show characteristic axial gradients in rate and total amount of reduction of permanganate, as indicated by the coloration of the protoplasm by MnO_2 or other products of the reaction. These gradients correspond with the physiological gradients indicated by other methods and by the development, growth-form and functional activities of the organisms.

The gradients are present, not only in each zooid of the hydroid colony and in each tentacle of the hydranth, but in each axial complex, *e.g.*, a compound branch, and in the colony as a whole, provided it is vigorous and growing. In general the rate and amount of reduction decrease basipetally. In the medusæ reduction is most rapid and greatest in amount in the marginal and oral regions, less in other subumbrellar regions and least in the exumbrellar region. Animals killed by other means before reaction with permanganate show no gradient, or in some cases vestiges, if newly killed. The conclusion is drawn that differences in rate and amount of reduction of permanganate, as indicated by the coloration, may, with proper precautions, be used as an indicator of differences in fundamental physiological condition in different regions of an organism.

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NOTES ON SOME PROBLEMS OF ADAPTATION: 4. THE PHOTIC SENSITIVITY OF OGILBIA.[1]

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Parker ('09) has commented upon the curious fact that although so primitive a chordate as ammocœtes exhibits a well-defined integumentary sensitivity toward light, marine fishes as a class seem devoid of this type of irritability, which is also absent in *Amphioxus* (Parker, '08; Crozier, '17). Recently Jordan ('17) has described the first recognized instance of photic sensitivity resident in the skin of a marine teleost, the hamlet (*Epinephelus striatus*). I wish now to record a second example of this type of sensitivity in a marine fish, *Ogilbia* (*Brosmophycis*) *verrillii* Garman.

The case of *Ogilbia* is of peculiar interest. The Brotulidæ, to which group *Ogilbia* belongs, are for the most part deep water fishes, but include several forms occurring in warm shallow situations on the Pacific and on the Atlantic shores of America. They seem to represent the ancestral type from which may be traced the evolution of the blind brotulids of the Cuban caves (Eigenmann, '09). The behavior of *Ogilbia*, concerning which very little has been known, should in consequence be a source of important information bearing upon the derivation of the related cave forms.

Ogilbia verrillii (Garman, '00) was first collected at Bermuda by Verrill. According to Eigenmann ('09, p. 187)—who, curi-

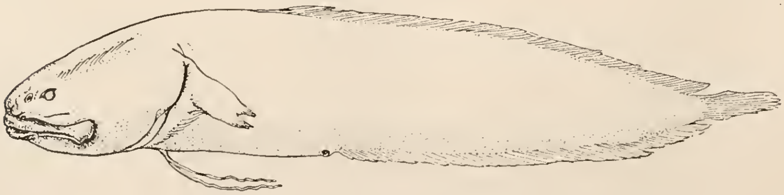

FIG. 1. *Ogilbia verrillii* Garm. (× $2\frac{1}{3}$). Note the relatively minute eye.

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[1] Contributions from the Bermuda Biological Station for Research, No. 130.

ously enough, in his review fails to mention the significant occurrence of *Ogilbia* at Bermuda—other species occur (*O. ventralis*) "in rocky pools about the Gulf of California and at La Paz," and (*Brosmophycis cayorum*) "on a shoal covered with algæ at Key West." My own specimens of *O. verrillii*, obtained singly and at scattered intervals, were secured from widely separated points about Bermuda—along the shore of Flatts Inlet, on the north side of Dyer Island, in Fairyland Creek, and in Tucker's Town Bay. In each case the habitat was at about low water level, either under a rock of some size or else in a dense mass of reddish and brown algæ. The length of these individuals was not over 4.5 cms. In color they were brownish yellow, speckled with minute red dots; there was little indication of counter-shading. They are not vigorous swimmers. When exposed to bright sunlight, by the sudden overturning of a rock slab, they seemed stupefied, and in several instances were easily secured by hand.

I wished to examine the behavior of these fishes toward light, and although but six specimens were available for experimentation, at different times from April, 1916, to December, 1917, the observations made seem adequate for certain purposes.

These fishes were intensely reactive to sunlight. If placed in an aquarium with one end illuminated from above, they remained almost stationary in the shaded portion, and if caused to swim toward the lighted area they turned back almost instantly when the anterior end came into the light. With direct sunlight, temperature 26° C., the reaction time averaged 1.3 seconds. Rheotactic response was also well marked, the animals heading in the usual way into a current. Taking advantage of this response, by appropriately directing a mild stream of water into a circular dish, it was possible to induce swimming movements without touching the fish.

Stimulated in this way, an *Ogilbia* would swim slowly up to the edge of a sharply defined sunbeam and remain stationary in the shade. Comparatively violent, undirected, swimming could be induced by suddenly illuminating the whole dish. Swimming movements ceased almost immediately when the light was obstructed. *Ogilbia* is very strongly thigmotactic and in most cases settled in the angle between the bottom and the wall of an aquarium.

With two specimens it was possible to remove the eyes and to keep the animal alive for about 24 hours thereafter. The eyes are quite small (Fig. 1) and burning operations were unsuccessful, two fishes being killed in attempts to obliterate the eyes in this way. The eyeless fishes were also found to be sensitive to light, and to orient, with some precision, away from it. The reaction time to direct sunlight was, in the one case measured, about 3 seconds.

Even with normal specimens, it was possible to induce weak swimming by illuminating merely the hinder half of the body.

There seems consequently no reason to doubt the occurrence in *Ogilbia* of a true photic sensitivity of the skin—a result of special significance in view of the integumentary photic sensitivity found in *Amblyopsis* (Payne, '07), and of the fact that the blind Cuban brotulid *Lucifuga* (Eigenmann, '09, p. 199) seems probably to be photonegative (although the data are scanty). This type of irritability is rather characteristic of cave vertebrates, and its presence in *Ogilbia* is perhaps susceptible of a significant interpretation. In Eigenmann's view, the blind cave fishes *derive* from types originally photonegative, living in darkness under stones along "coral" reef shores, which survived a process of adaptive adjustment to fresh water and to cave environments conditioned by gradual elevation of the land.[2] It is frequently assumed that special modes of behavior, notably those dependent upon enhanced photic and tactile irritability, represent new developments determined in relation to blindness and to the conditions of cave environments. Hence it is important to recognize that even a manifestation so rare among marine fishes as skin sensitivity to light, is adequately exemplified in a teleost, not cave-inhabiting, but with eyes seemingly degenerate and on other grounds assigned as representative of the ancestral origin of typically adjusted cave forms. The state of pre-adaptation thus indicated is reflected also in connection with tactile irritability (Crozier, '18).

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[2] It may be mentioned that I have several times made search for fishes in cave pools at Bermuda, but have never encountered fishes in them, save where obvious communications led to outside water.

Summary.—*Ogilbia verrillii*, shore-living brotulid and close relative of blind brotulids of Cuban caves, has relatively small eyes and is decidedly photonegative in its behavior. Its skin is sensitive to light, a condition thus far recognized in but one other marine fish; this is taken to be of significance for the conception of preadaptation.

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NOTES ON SOME PROBLEMS OF ADAPTATION: 5. THE PHOTOTROPISM OF LIMA.[1]

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The monomyarian lamellibranch *Lima* is said (Kafka, '14, p. 393) to be "photoptic," *i.e.*, reactive to light but not to shadows. The statement may be applied to the species of *Lima* found in some abundance at Bermuda, but there are certain additional features about the photic responses of this animal which merit a more extended notice.

Polimanti ('12) has distinguished three types of locomotion in *Lima hians:* (1) by the use of the foot as a lever; (2) by closure of the valves and retraction of the tentacles (Butler, '91); (3) by the expelling of water through the siphons. The first of these types may however have some complex aspects.

Lima was usually obtained near low water, around the edges of large stones, and among smaller stones piled together loosely, in bays where a certain amount of silt was being deposited; but was also encountered on the reefs, in crannies between corals, and sometimes among colonies of *Ecteinascidia.* It is decidedly photonegative in its behavior, although this activity may be lost after some days of laboratory captivity in open dishes. The edge of the mantle is beset with long tentacles, which are only very slightly adhesive toward wood or glass, but upon which carmine particles stick and accumulate readily. When touched, these tentacles react individually, by a vigorous and complicated retraction, but the reaction time is curiously long (about 1 minute, after a vigorous prod). They are reactive also to strong currents of sea water, and respond vigorously to the local application of a gentle stream of fresh water. At rest, the long white tentacles are widely extended (for a good figure, cf. Nutting, '19, p. 93). The tentacles are quite sensitive to weakly acid solutions; and, after the animal has been for a time in darkness, illumination seems to cause a variable number of them to curl and contract, but not sharply; they do not themselves appear locally sensitive to light. Stimulation of a tentacle by weak acetic acid in sea water caused the particular tentacle to retract instantly, and the animal to immediately swim violently away.

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[1] Contributions from the Bermuda Biological Station for Research, No. 131.

In swimming, *Lima* has open to it a variety of possibilities. It may progress while lying on either side, after the manner of *Pecten*, or it may swim with the medial plane in the vertical position, the animal being out of contact with the substratum save between "steps." The "steps" may in either case be made in one of several ways—either toward the gape of the shell, as with *Pecten*, in which event the mechanics of the swimming act is similar to that of *Pecten* (Vlès, '06; Bauer, '12); or else toward the hinge, by squirting water from between the valves. The mantle fold is relatively wider than in most Pectinidæ, and is very mobile; at rest, it beats with a rhythmic pulsation, doubtless functionally homologous with that known in some other lamellibranchs (Redfield, '17), but different in one regard: in *Anodonta* and in other clams the opposite mantle-flaps beat synchronously, whereas in *Lima* they unmistakably pulsate in opposite phase, alternately.

The *Pecten*-like mode of progression is the one usually adhered to, whether the animal be swimming vertically or on one side (and by actual count it was found that either side is used, indifferently). This is almost the only type of swimming induced by photic stimulation, the other mode of progression, in the direction of the hinge, being slower and made up of very short "steps."

When a previously darkened *Lima* is illuminated by horizontal sunlight, it turns the gape away from the light and swims, usually with the valves vertical, in a straight line away from the light. There is exhibited a definite and peculiar process of orientation. The long, mobile foot is thrust out between the valves, its free portion is bent sharply away from the light, and its distal end becomes adherent to the substratum. The foot then shortens longitudinally, so that, without moving its valves, the body is turned somewhat away from the light; several "steps" of this sort, perhaps three in all as a rule, result in an almost perfect alignment of the median plane in the axis of the light beam; the *Lima* then swims, *Pecten*-wise, in a straight line away from the light. The swimming act is not usually prolonged, perhaps 50 cms. representing the maximum distance covered before temporary exhaustion intervenes, but the orientation process is of a definite, clean-cut character and was previously unknown, so far as I am aware, among bivalves. It is a point of some interest that in a photosensitive form such as *Lima*, where structural conditions give the possibility of phototropic behavior, we do in fact find such behavior diagrammatically exhibited.

The process of body-orientation is usually as just described; but if light be directed upon the gape of a vigorous *Lima* it sometimes gives a violent somersault, turning completely through 180° by some mechanism which I was not able to analyze; the subsequent swimming is in the usual manner.

I tried to find out if the foot, so active in the commoner orientation process, were itself photosensitive. After various trials with local illumination I came to the belief that it was, but complete proof of this I did not secure.

It is especially when the animal is weakened by handling or by laboratory conditions that swimming on the side is found. When in good condition a *Lima* can swim for at least 25 cms. through the water without touching substratum.

The photonegativity of *Lima*, involving so curious a method of orientation, is obviously important in maintaining these animals in crannies and darkened clefts, under stones and among coral heads.

Summary.—The bivalve *Lima* is photonegative, and in vigorous individuals the response to illumination involves a true and accurate orientation of the body, with gape away from the light, by means of the long adhesive foot, which is itself perhaps photosensitive; orientation is followed by *Pecten*-like swimming, the valves being vertical.

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NOTES ON SOME PROBLEMS OF ADAPTATION: 6. RELATION OF LIGHT TO THE PIGMENTATION OF ASCIDIANS.[1]

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1. Experiments concerned with the re-formation of the test of the blue-black *Ascidia atra*, subsequent to its injury or partial removal, have demonstrated (Hecht, '18a, p. 236) that "the newly formed test material is pigmented in the usual way," even in the case of animals operated upon at night and maintained during regeneration in complete darkness. Since, "moreover, a new, pigmented test will form on the right face of the animal under the intact, opaque, old one, when the latter has been accidentally separated from the ectodermal surface which secretes it," Hecht concluded that the characteristic pigmentation is not due to the presence of light—in the sense, at least, that it cannot result from "a photic stimulus only." This conclusion is of interest, since it at first sight conflicts with some facts afforded by related species, and also with facts which may be made out from the distribution of *A. atra* itself in nature. Hecht's experimental result I can fully confirm, but inasmuch as pale, relatively unpigmented individuals are to be encountered in dark situations, the relation of the illumination to pigmentation seemed far from clear.

It might be assumed that if, in the normal course of development, pigmentation were simply a qualitative result of metabolic events, the quantitative course of transformations leading to pigment deposition in the test might be determined, once for all, by illumination early in the ascidian's life; if this were correct, experiments upon adult animals might be quite incompetent to decide whether or not light has anything to do with pigmentation—and effects substantially of this category are not unknown (Goldfarb, '10).

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[1] Contributions from the Bermuda Biological Station for Research, No. 132.

2. Owing to the nature of its normal mode of occurrence, which is most frequently in bright, sunlit situations in shallow water, *A. atra* is rendered quite conspicuous by its dense blue-black coloration—so much so, in fact, that some observers (cf. Mortensen, '17) have not hesitated to suggest the operation of "warning" coloration in this connection. In view of this fact, and of the direct relation, in *Ascidia*, between pigmentation and blood metabolism (Hecht, '18a), the finding of pale individuals in dark situations required further analysis.

Simple ascidians having transparent or translucent bodies are commonly found to frequent dimly illuminated spots. Examples are given by *Ascidia curvata*, quite transparent and at Bermuda found almost always under fair-sized stone slabs, although taken by me also among masses of the alga *Valonia*, the dead cells of which are closely imitated by it (!); by *A. californica* (Ritter and Forsyth, '17), from the undersides of rocks; and by *Ciona intestinalis*, "especially given to clinging to the undersides of floats, buoys, and the like." The original assumption of a darkened habitat by these forms is probably due to the fact thar the ascidian larva, at first photopositive, becomes sooner or latet negatively heliotropic, before attaching; in different species this reversal of behavior with respect to light may be much delayed, or may even not appear at all. (In another place I shall discuss experiments concerned with the behavior of the tunicate tadpole.) It is of interest to note that the adult *Ciona* is quite reactive to light, in a sensory way, and also that in the laboratory these animals die rather quickly in diffuse light, although in dim light or darkness they are able to live for some days (Hecht, '18a); whereas *A. atra*, adult, is insensitive to photic excitation (Hecht, '18b; Day, '19).

Very pale examples of *A. atra*, however, were, as stated, found under stones in various places at Bermuda. Some of these were carefully gathered, and used in the following experiment:

"Aug. 1 to 7, 1918. Under stones on the shore of Trunk Island, Harrington Sound, 4 very pale *A. atra* secured; blue-black pigmentation was apparent only at the very tips of the siphons. These were kept in laboratory dishes lighted by a northwest window, some little direct sunlight falling upon the animals late each afternoon. The tunicates were perceptibly blacker on the second day, and rather dense black by the seventh day."

The experiment was several times repeated. The individuals concerned were 1.2 to 2 cms. long.

From such results it might appear that in the case of *A. atra* light has indeed a direct effect in producing dark pigmentation, as it undoubtedly has in certain molluscs (cf. List's observations, 1902, on the superficial pigmentation of mussels; these observations I can confirm from experience with the Bermudan littoral *Modiolus*). But it is known that a number of tunicates change color under laboratory conditions (Caullery, '95; cf. also Holt, '14). Accordingly, I repeated the above experiment, with the difference that the animals taken from under stones were immediately placed in darkened collecting buckets, and in the laboratory maintained in aquaria in a dark room. By the sixth day, these individuals were as dark as the similar ones exposed in the laboratory to illumination. Hence it cannot be decided from such experiments whether or not light has any direct significance for pigment-deposition in the test of *A. atra*.

3. A possible explanation was sought in another direction. Every *A. atra* found on the under side of a stone slab was relatively small, none being more than 4 cms. long. Individuals of this length collected from reef rocks, wharves, buoys and floats, moreover, were comparatively much more robust. As an index of better growth, the thickness of the test on the right side of the body was measured in several of these. This thickness, in the normally situated ascidians, amounted to 2.0 ± mm.; whereas in those living under stones the thickness of the test was but 0.8 mm. Since it is fairly certain that the blue-black granular pigment of the *A. atra* test derives from metamorphosed vanadium-containing blood cells (Hecht, '18c), and is essentially an excretory product (Crozier, '16), as seems probable for some other ascidian pigments also (Pizon, '01), it is not unreasonable to suspect that malnutrition may be in these instances responsible for faulty pigmentation. Fuchs ('14) observed, in laboratory cultures, that the siphons of young Cionæ lengthen remarkably in

proportion to the available food material, and was able to correlate this finding with the forms of *Ciona* in different natural environments. *A. atra* from among piled stones was noticed (before Fuch's paper was known to me) to have siphons very short in comparison with those of the more robust specimens from open situations. The view is, therefore, permissible that relatively deficient metabolism is in this instance responsible for subnormal pigment deposition.

The experiments of Miss Johnson ('13) regarding the pigmentation of amphibian larvæ showed the kind of materials ingested, rather than the absolute amount of food, to be important for pigment formation. In *Ascidia* we are not dealing with a melanin, however, but with a quite different type of pigment. Unfortunately, one is not able to decide as to whether the larvæ ultimately producing the pale *A. atra* located under littoral stones came in the first place to assume such sites in an accidental way, or were carried there and, because of some deficiency in vigor, failed to escape, or because they endured too long (or not long enough) in the larval phase and became attached at a time of day unsuitable for the operation of photic control of their movements.

The larva of *Ecteinascidia*, as I shall elsewhere describe, is normally liberated on a falling tide, and the duration of its free-swimming phase, together with its modes of response, are so adjusted to the tidal rhythm that in most cases a new individual (in this latter species, resulting in a colony) is laid down at about low-water level; the exact location of the site of attachment depends in an interesting way upon developmental changes in the phototropism of the larva. (Parallel changes have since been described by Grave, 1920, in the larva of *Amaroucium;* but the ethologic correlations, fairly clear in *Ecteinascidia*, have been as yet incompletely set forth for *Amaroucium*.) Should similar phenomena appear in the comportment of the larva of *A. atra*, some transforming individuals might become too photonegative, or might settle down at night, in either event perhaps becoming affixed in a dark cranny among stones.

4. Definite proof that darkness as such has no control over

the pigmentation of *A. atra* seems to me indicated by the finding of well-grown and densely black specimens in small caves practically shut off from the light. A few such were discovered with the aid of an electric torch in cavelets having but a small opening to the water. And from the under surface of a submerged rock, undercut in such a manner as to be completely sheltered from the sun, I later secured a group of fair-sized individuals with a quite normally black appearance.

5. *Summary.*—Pale, translucent individuals of the blue-black *Ascidia atra*, a species normally occurring in sunlit places, are found in darkened situations under stones. They resemble, in the absence of test-pigment, ascidians of other species habitually living in shaded spots. In spite of the fact that such pale *A. atra* may quickly blacken if placed in sunlight, it is pointed out that the absence of natural pigmentation cannot be regarded as due to darkness, but is probably a consequence of faulty nutrition.

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NOTES ON SOME PROBLEMS OF ADAPTATION: 7. REGARDING THE PIGMENTATION OF STICHOPUS MŒBII.[1]

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The large holothurians of the genus *Stichopus*, which are quite abundant at Bermuda, live for the most part in fairly shallow water and upon grass-free bottoms of sandy mud. The majority of the individuals, but not all, are quite darkly pigmented. They are consequently open to the view of fishes and of other predaceous creatures, their coloration being in the great majority of cases very far from "concealing" in character. I was curious to learn if this lack of concealment was in any way related to size, or to the presence of some repugnatorial property. These holothurians do not possess Cuvierian organs.[2]

About 70 per cent. of the individuals are dorsally of a deep brown or even blackish hue, some 20 per cent. being light brown, with a distinctly yellowish cast, and the remaining 10 per cent. very pale brown with a pinkish shade. About 75 per cent. of the two kinds of lighter-colored specimens show a series of circular dark blotches, located at the bases of the scattered podia. A small proportion are irregularly mottled or blotched with areas of dark pigmentation. The pale spotted individuals are in many instances very difficult to detect, especially if located among algæ and corals on an inclined shore; on a bare mud bottom they are more easily seen. The amount of pigment in the integument bears no definite relation to age (size).

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[1] Contributions from the Bermuda Biological Station for Research, No. 133.

[2] I have vainly sought for instances in which the well-developed Cuvierian organs of *Holothuria captiva* (Crozier, '15) might be seen in action (cf. Cuénot, '98). This species sometimes lives, under stones, in association with the large annelid *Hermodice carunculata*, which is armed with powerful groups of long, sharp setæ; but I have never seen evidence of disturbance of the holothurian by the annelid. The tubes of the Cuvierian organ when discharged in air and received on filter paper (or on the tongue) are never acid in reaction; apparently their sole utility is in connection with their tensile strength and adhesive power.

In view of the absolute lack of any concealing behavior practiced by these creatures, I tried at various times to feed pieces of the skin to fishes ("squirrels," yellow grunts, gray snappers, and other common shore forms, including the hamlet). Such trials were invariable unsuccessful, the flesh being refused. And, as Minchin ('92) long ago found, the intestine and respiratory trees are likewise rejected as food.

It is of interest to note that, in spite of such evidence of "unpalatability,"—which may perhaps be supplemented from Bartsch's ('17) account of the use of "holothurians" by the natives of Guam in driving *Octopus* from its "nests,"—holothurians are nevertheless occasionally seen in the rôle of hosts for erratic ectoparasites, which in some instances eat from their skin. Chatton ('09) found an ascidian attached to and growing on the integument of *Holothuria*, and I have seen a young actinian (*Condylactis*) firmly affixed to the integument of a *Stichopus*. Quite apart from such molluscan forms as *Stylifer*, one species of which I have found in considerable numbers on *S. mœbii*, I have records of an annelid (an unidentified species of *Odontosyllis*) and of a polyclad worm, *Leptoplana*, feeding on the skin of *Holothuria surinamensis;* the gut of the worm was in each of these cases filled with the bright green integumentary pigment of the host. So that it is difficult to postulate for these animals the possession of any generalized repellent secretion. Plate ('16), also, has described a polynoid and a crab, both homochromic in coloration, living upon *Holothuria atra*.

On the other hand, *Holothuria surinamensis* has on several occasions been found bearing clear evidence of injury. One specimen was picked up, lying in the open on a sandy bottom with a shallow gouge cut along the whole length of the body; in all probability it had been nipped by a crab, perhaps *Callinectes*. Later, three others were obtained, all injured in a similar way. In the absence of some repellent "flavor," it is difficult to understand why the holothurians, once bitten into in this way, should have been dropped. This particular species, as I have said, is devoid of Cuvierian organs, and in the mangrove creeks where it notably abounds it comes to the surface only at night, being buried during the day about the roots of sea weeds. Its skin, incidentally, is covered by a slimy coating of mud-particles, tending to give it a concealing hue; whereas *H. captiva*, well equipped with Cuvierian organs, but living under stones, is of a brilliant dark green color, unobscured with mud.

In feeding tests, the flesh of *H. surinamensis* was rejected as vigorously as was that of *Stichopus*.

It seems on the whole quite possible that some repugnatorial property is present in the secretions of the holothurian skin. The repellent power of the skin of the nudibranch *Chromodoris zebra* has been amply demonstrated (Crozier, '16, and subsequent work), yet I have found ostracods (*Cypris*) creeping freely over its skin; and while the blue-black *Ascidia atra* is rejected, whole or in fragments, by fishes I have tested, certain polyclads, nevertheless, feed freely on its surface (Crozier, '17). It is not inconceivable that the slimy quality of the holothurian flesh, particularly as developed when the body wall is cut, is in itself a sufficient repellent. I have never succeeded in inducing aquarium fishes to swallow swollen pieces of gelatin, although in this case a specific repellent action seems improbable.

The adult *Stichopus* is, at all events, practically immune from the grosser kinds of attack. The only dead or injured specimens I have seen were killed by heavy rain-fall on shallow shores at low tide, or else by the sun when left by the tide on an exposed beach. The flesh of these animals is not an acceptable article of diet to fishes or to octopus, and whether this immunity be due in part to specific repugnatorial secretions, for which there is some evidence, or to some physical property of the flesh, or to the large size of the individuals, the feature which for present purposes I regard as significant is this: although efficiently protected, as it seems to be, the coloration of the species exhibits a wide range of variation, such that in some instances it might be thought "warning," in others decidedly "concealing." The conclusion is difficult to avoid, that the "conspicuous" type of pigmentation is only associated with immunity from attack in a secondary, and in a sense an accidental, manner. The flesh of the paler, spotted, and as I believe, on the whole better concealed, individuals, is no more acceptable as food than that of the blackish specimens. The different degrees of pigmentation are determined, so far as one may judge, by quantitative differences in the capacity for producing the single type of pigmentary substance concerned. So far as available evidence permits one to say, it is as reasonable to hold that the immunity of *Stichopus*, however achieved, permits its pigment-forming power to develop unhampered by selective restraint, as to believe that a kind of "warning" coloration is being elaborated selectively. In this respect the diverse types of coloration found in the efficiently protected snail *Onchidium* (Crozier and Arey, '19) provide an interesting parallel. And in *Chromodoris* (Crozier, '19), as I shall subsequently describe at length, the evidence is much more decisively of the same import. Questions of this sort, particularly as concerned with the matter of so-called "warning" coloration, have been, in my belief, on the whole so inadequately dealt with that even partially complete observations seem worthy of presentation.

Summary.—The coloration of *Stichopus mœbii* is quite variable, in some individuals probably of a concealing tendency, in others, the majority, of a sort which makes these animals in nature pronouncedly conspicuous. Perhaps conditioned by the bulk of the animals, possibly by the presence of a repugnant quality of the flesh, such as leads to the rejection by fishes of fragments offered as food, a decided immunity from gross attack is, at all events, achieved. There is no more reason to regard the conspicuous type of pigmentation as a warning agent than there is to consider it the unchecked expression of innate metabolic activities.

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NOTES ON SOME PROBLEMS OF ADAPTATION: 8. CONCERNING "MEMORY" IN ACTINIANS.[1]

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By a number of writers (cf. Parker, '17) it has been believed that certain animals of intertidal habitat may continue for a time to exhibit rhythmic movements, either of locomotion or of opening and closing, when they have been removed to aquaria and maintained under more or less constant conditions, and. further, that such periodic movements may be synchronous with the rise and fall of the tide. Unfortunately, many of the instances brought forward, *e.g.*, among the actinians, have been found upon closer scrutiny to yield no real evidence of a persisting "memory" of tidal events.

One of the commoner Bermudian sea-anemones, the red *Actinia bermudensis* Verr., occurs abundantly in caves along the shore and in other mud-free situations open at times to a moderate surf, but almost always between the tide lines. When the tide falls, the actinians retract the tentacles and constrict the column-sphincter. Commonly these sea-anemones hang upside down from the under faces of stones, but they are also abundant upon tiny ledges in the walls of small caves—in any event, occurring in situations such that they are rarely left in a tide pool, but are, on the contrary, freely exposed to the atmosphere by the retreating tide.

The much larger actinian *Condylactis passiflora* usually lives beneath low tide, with the column imbedded among stones; at extremely low tides, however, some individuals may be left a little distance out of water, and it is noteworthy that these do not retract the tentacles and contract the column, but instead hang down limply with the tentacles extended and the column passively stretched by the weight of the fluid in the interior cavity.

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[1] Contributions from the Bermuda Biological Station for Research, No. 134.

The correlation, therefore, between a normal intertidal habitat and the practice of so contracting the body as to conceal the tentacles, decrease the surface for evaporation, and present a smoothly rounded exterior surface, is decidedly worthy of study. A variety (*prunicolor* Verr.) of *A. bermudensis* was found in bays on the south shore of Bermuda not under stones, but in small tide pools open to the sun, and also within the tidal zone. This variety is of a brownish hue, whereas the ordinary *A. bermudensis* is deep red. The color difference, apparently the only feature separating the two forms, was constant, and was even seen in young taken from the gastrovascular cavities of adults; the young of the red forms were always pink or scarlet, those of the brownish forms a light prune-color. The occurrence of such color differences has also been noted in related forms. Elmhirst and Sharpe ('20) state that the deep red *Actinia equina* becomes brown in the laboratory, and that the change "breeds true"—a remarkable observation, if correct, and one which should be followed up; it corresponds well with the conditions known in *A. bermudensis*. The red pigment of the latter species, readily extracted by acetone, is changed to a brownish hue by sunlight. The brownish anemones close up in the usual manner when the tide falls.

A. bermudensis reproduces "viviparously" throughout the year. Experiments could therefore be made with young actinians—"born" in the laboratory or taken from the cœlenteron of adults—which had never themselves been directly exposed as free individuals to the action of the tidal rhythm.

When the adults were removed from the natural location at low water and placed in an aquarium, they immediately relaxed and the tentacles were extended. After variable periods, of several hours, in non-circulating water, they closed again, although still completely covered by water. In a group of twenty individuals which were on one occasion (in January, 1917) placed in an aquarium, the period elapsing before closure was about three hours. These particular actinians all came from very nearly the same level above low water, and since most of them after a time opened again, I was led to look for a tidal rhythm. In point of fact, however, it is more than doubtful that any such rhythm exists. The behavior of different specimens is anything but synchronous in the subsequent periods of opening and closing, and there is no tidal periodicity evident,—except perhaps in the matter of remaining expanded for about three hours after being immersed in water. But this interval is much the same regardless of the period of antecedent exposure; for specimens taken from the rock just after being left bare by the receding water are in this respect indistinguishable from those removed at the natural termination of the interval of exposure. After a sea-anemone has contracted, in still water, it may remain so for days, or it may after a time open "spontaneously." At any time it may be caused to expand by a faint local agitation of the water in its vicinity. In this behavior *A. bermudensis* was very similar to *Metridium* as described by Parker ('17). Sea-anemones attached to the rocks could always be caused to open by splashing over them a small amount of water; in this kind of response is to be seen the explanation of a sort of "anticipatory" expansion sometimes seen on the shore, wave-dashed droplets of spray being sufficient to induce some relaxation of the column-sphincter before the animals are actually covered by the sea.

Young *A. bermudensis* "born" in the laboratory aquaria exhibit precisely the same phenomena. In a group of six, one or two individuals may be found at any time tightly contracted, the others being in various degrees of expansion. But this behavior is not synchronous. One such group was rather carefully watched at intervals during 3 weeks. No rhythm of contraction was detectable in the individual behavior of these animals. Nevertheless, if they be taken from the water for a short time and then replaced, such young individuals promptly open and remain extended for about as long as do the adults under similar treatment, but after this they contract for variable periods.

Hence the rather fixed interval of expansion consequent upon immersion in sea water, although it agrees fairly well with the period of natural submergence, can hardly be ascribed to the establishment of a tidal habit; for the young actinians inexperienced in the matter of tides go through the same performance as the adults. Of course, it might be supposed that during the period of low water changes in oxidation or the accumulation of metabolites within the cœlenteron might rhythmically influence the behavior of the embryos and juveniles. But the important point is the significance of purely mechanical stimuli, derived from motion of the water, for the relaxation of the column-sphincter. In a contracted sea-anemone, small or adult, expansion could always be secured by gentle movements of a glass rod in the nearby water. The necessary conclusion is that in the absence of such mechanical stimulation the actinian contracts; the latent period for this response is rather long. There is no evidence of tidal "memory."

Summary.—The behavior of *Actinia bermudensis* is considered with reference to its intertidal habitat. It is likely that the habit of contracting when uncovered by the sea is important for the maintenance of the species between tidal limits, but there is no evidence for persistence of the rhythm of contraction and expansion in the absence of the tidal rise and fall. After a period of about three hours in still water, the actinians close; they can at any time be caused to expand by agitating this water. Young individuals, never directly exposed to the tidal cycle, behave in the same manner.

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BIOLOGICAL BULLETIN

ANOTHER MALE COPEPOD OF THE GENUS SALMINCOLA FROM THE GILLS OF THE CHINOOK SALMON.

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The only male copepod known to exist in the genus *Salmincola* of the family Lernæopodidæ is that of *Salmincola edwardsii* (Olsson) Wilson, which parasitizes the brook trout *Salvelinus fontinalis* Mitchill of many of the states of the middle West and East. This male has been described and figured by the writer in the BIOLOGICAL BULLETIN, Vol. XXVII., for August, 1914. During the latter part of the summer of 1919, the author obtained large numbers of another species of *Salmincola*, namely, *Salmincola beani* Wilson, parasitic on the gills of the chinook salmon *Oncorhynchus tschawytscha* Walbaum of Green River, Auburn Washington, and a careful search amongst these parasites, resulted in the discovery of three mature males.

The size of these new male *Salmincola* is much larger than that of *Salmincola edwardsii* (compare Figs. 1 and 3 with 4). The former (Figs. 1 and 3) measure about 1.0 mm. in length and about 0.17 mm. in width in the region of the reproductive organs; whereas the latter (Fig. 4) are about 0.7 mm. long and 0.1 mm. wide in the region of the gonads. Otherwise their structure seems to be similar.

The organs which are of particular interest and importance in the male are the reproductive organs. These are paired structures, located laterally in the posterior region of the body (Fig. 1, *r*), between the digestive tract and the body wall. They consist essentially of the following three main parts: (1) the testes (Fig. 2, *t*), (2) the coiled vas deferens (Fig. 2, *v*), and (3) the pear-shaped spermatophores (Fig. 2, *s*). The spermatozoa are manufactured in the testes, from which they are conducted into the vas deferens, and from here they are then conveyed into the spermatophores, where they are stored until the male is ready to fertilize the female. Located near the spermatophores, on the ventral side of the body are two openings, the ejaculatory pores (Fig. 2, *e*), through which the spermatophores may be extruded.

Fertilization in the Lernæopodidæ occurs shortly after the attachment of the free-living copepods to the host. In *Salmincola edwardsii* it takes place about two and one half to three weeks after attachment, and in the species under consideration, *Salmincola beani*, although the time of fertilization has not been accurately determined, it, in all probability, takes place after a similar lapse of time. When the copepods are ready for fertilization, the male is about one third or one fourth the size of the female.

Prior to fertilization the males and females hang side by side on the gills of the host, being attached by the so-called attachment filaments. In the male, the attachment filament remains long and tubular, with an enlarged bulla-like structure at one end which is firmly fastened in the flesh of the gill, while the opposite end is slightly enlarged and to it are attached the terminal claws of the male's second maxillæ. In the case of the female, the tubular portion of the attachment filament disintegrates shortly after the attachment of the female to the host, so that only the bulla-like portion remains for the attachment of the parasite.

In order to effect fertilization the male must seek out a female, release his hold on the gill, and then in some way become attached to the posterior margin of the female's body, in the region of the genital pores. The specific manner in which this is accomplished is as follows: When the male reaches maturity he undergoes circling movements. If he meets a female in the radius of his explorations, he grasps her body with the terminal claws of his free maxillipeds and at the same time releases his hold on the attachment filament. The male now creeps over the body of the female until he reaches the neighborhood of her genital pores and here he attaches himself and remains until after fertilization. In Fig. 1 the male is seen attached to the middle of the body of the female, while in Figs. 3 and 4 the male is at the posterior margin of the female, in the vicinity of the genital pores ready to fertilize her.

Fertilization is accomplished by the male bending the posterior portion of his body towards the genital pores of the female. Then the male extrudes his two spermatophores through the ejaculatory pores, and by means of the second maxillæ he attaches these near the genital openings of the female. The spermatozoa soon wander through these openings and become stored in the spermatheca of the female. After the migration of all the spermatozoa, the spermatophores shrivel up and come to look like transparent, shell-like, yellowish spheres. Very shortly after fertilization the male dies, while the female lives on and develops a great many young, which are capable of carrying on the life-history of the species. In order to insure fertilization, it is by no means uncommon to find that many of the females have been fertilized by more than a single male.

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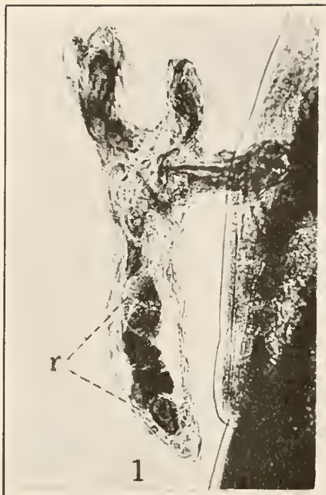
EXPLANATION OF PLATE I.

1. Male *Salmincola beani* Wilson, attached to middle of the abdomen of a mature female. The reproductive organs (*r*) can be distinctly seen. × 65.

2. An enlarged photograph of the reproductive organs of the male seen in Fig. 1. *t*, testis; *v*, vasa deferens; *s*, spermatophore; *e*, ejaculatory pore. × 183.

3. Male *Salmincola beani* Wilson in position near the genital pores of the female. × 65.

4. Male *Salmincola edwardsii* (Olsson) Wilson attached to genital region of female. × 65.



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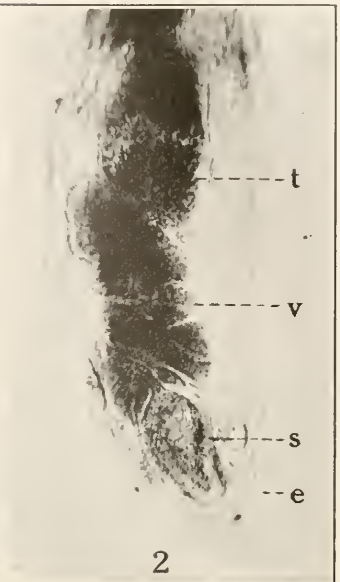

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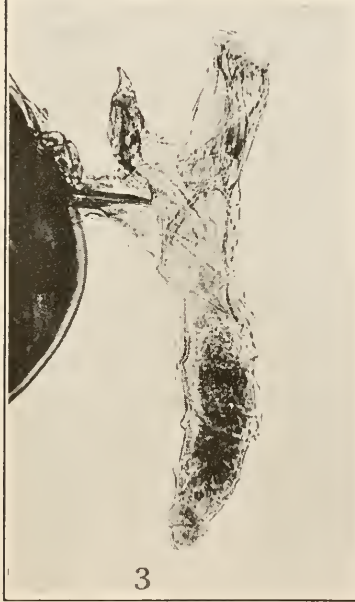

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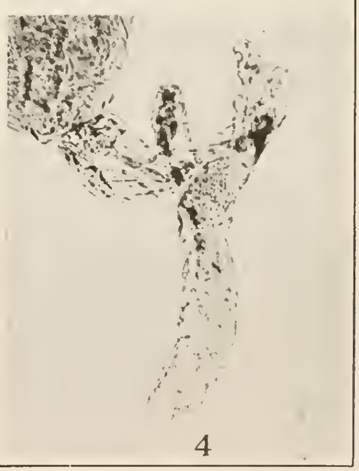

4

NATHAN FASTEN.

STUDIES OF FERTILIZATION, X.

THE EFFECTS OF COPPER SALTS ON THE FERTILIZATION REACTION IN ARBACIA AND A COMPARISON OF MERCURY EFFECTS.

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1. INTRODUCTION.

Copper salts are known to have a profoundly injurious effect on many organisms even in high dilutions. They are also known to form compounds with ferments and proteins (on one side of the isoelectric point). It might therefore be expected in advance that they would have very definite effects in the delicate reactions of fertilization, which might be used advantageously in the analysis of these reactions, the precise character of which is still a matter of dispute.

The results reported were obtained in the summers of 1920 and 1921 at the Marine Biological Laboratory at Woods Hole. They have led me to the conclusion that the fertilization reactions in the sea-urchin are due primarily to activation of a ferment-like substance contained in the cortex of the egg which is presumably identical with the fertilizin of my earlier studies. The method of approach is quite new in fertilization studies, but has been previously used in ferment studies, as I discovered after the conclusion of my experiments. Certain points of comparison between inactivation of ferments by salts of heavy metals and inactivation of the fertilization reaction were found.

As soon as we adopt the conclusion that fertilization is essentially activation of a substance, contained in the cortex of the egg, by the spermatozoön (Lillie, 1914, 1919), the way is open for study of the properties of this substance by means of inhibitors of fertilization in general. If we were to determine the qualitative and quantitative range of such inhibitors we would be in possession of a list of properties of the postulated substance that should enable us to classify it very closely. The present paper is a contribution to this program.

II. Methods.

The eggs of the sea-urchin, used in these experiments, should be washed in two or three changes of sea-water to rid them of blood or tissue secretions, and they should not be used for more than about three hours after washing. The quantities used should be small and constant for all experiments of a set; as a practical matter not more than two drops of eggs from the layer on the bottom of the container to 10 c.c. of sea-water. In more delicate experiments egg concentration should be expressed as number of eggs per c.c. of sea-water.

Sperm concentration may be expressed in terms of dilution of the dry sperm as I have done previously. For instance, one drop (0.1 c.c.) of dry sperm may be added to 25 c.c. of sea-water, and one drop (0.04 c.c.) of this suspension used to fertilize one drop of eggs in 7.5 c.c. sea-water. This may be expressed as one drop of 1:25:7.5 c.c. Such an insemination is abundant but not "heavy." It is equal to about 1.5 "units sperm concentration" of study VIII. Sperm concentration is given for all the experiments. A 1:25 sperm suspension should be prepared immediately before using and should not be used for more than about five minutes, as its fertilizing power diminishes rapidly. The main point is to make sure that the inseminations of a series are identical or comparable.

A solution of 1 per cent. by weight of c.p. copper chloride in distilled water was made; 0.1 per cent. in distilled water was made from this, and the various concentrations actually employed in the experiments were made immediately before each experiment by adding a definite number of drops of the 0.1 per cent. solution to 100 c.c. of sea-water, using a pipette graduated in hundredths of a c.c. on which the actual amount added was read off. The strength of copper chloride most commonly employed was 5 drops of 0.1 per cent. in 100 c.c. of sea-water. The readings of the 5 drops ranged around 0.19 c.c.; in round numbers this was calculated in as 0.2 c.c. and the amount of $CuCl_2$ in such a solution is accordingly given as one part in 500,000. This is approximately 1/67,000 N, but on account of the alkalinity of the sea-water the effective concentration must be somewhat less.

In some early experiments a stock solution of copper sulphate in sea-water was used. The strength of this declined rapidly and it was used only in experiments 1 to 9. Its physiological effects are the same as copper chloride.

For each experiment there are two standard controls, viz.: (1) the *copper control*, *i.e.*, an identical insemination in copper chloride of the same concentration, and (2) *gamete control*, *i.e.*, an identical insemination in sea-water. Special experiments had in addition their special controls.

III. Experiments.

1. *The Phenomenon of Copper Inhibition. Relations between copper concentration and sperm concentration.*

The presence of one part of copper chloride in 500,000 parts of sea-water will completely inhibit fertilization of the eggs of *Arbacia*. Previous exposure of the gametes to the action of the copper chloride is not necessary for this result; if eggs and sperm are dropped simultaneously into the copper-containing sea-water, and mixed at once, no reaction occurs, though the sperm are as active as in normal sea-water. With most batches of eggs the inhibition is complete even when the amount of sperm used is several times what is necessary to fertilize 100 per cent. of the eggs in normal sea-water.

Though the eggs may be bombarded by hundreds of spermatozoa each, they do not usually give the first beginnings of the fertilization reaction; but, if any egg does so, fertilization is carried through to completion in the copper solution, and the egg segments. The inhibition is thus an "all or none" effect, as is shown more fully beyond.

The relations between copper concentration and sperm concentration are given in Table I. If the concentration of the copper is much greater than one part of copper chloride in 500,000 parts of sea-water, the inhibition may be regarded as complete for all sperm concentrations.

TABLE I.

RELATIONS BETWEEN COPPER CONCENTRATION AND SPERM CONCENTRATION. Time of exposure before fertilization = 0. Percentages are of eggs that segmented.

In these experiments the eggs were fertilized with definite concentrations of sperm (horizontal lines) in sea-water containing copper chloride to the amount of 1 part in 2,500,000, one part in 1,250,000, etc., to one part in 416,666 (vertical columns).

Sperm Concentration.	Sperm Concentration Units. See Study VIII.	$\frac{1}{2,500,000}$ $CuCl_2$	$\frac{1}{1,250,000}$ $CuCl_2$	$\frac{1}{833,333}$ $CuCl_2$	$\frac{1}{625,000}$ $CuCl_2$	$\frac{1}{500,000}$ $CuCl_2$	$\frac{1}{416,666}$ $CuCl_2$	Control No $CuCl_2$	Number of Experiment.
One drop 1 : 25 : 7.5 c.c.	1.5	50%	7%	0	0	0		100%	Exp. 35
Five drops 1 : 25 : 7.5 c.c.	7.5	100%	7%	50%	0	0		100%	Exp. 35
One drop 1 : 25 : 7.5 c.c.	1.5			5%	0	0	0	100%	Exp. 36
Five drops 1 : 25 : 7.5 c.c.	7.5			2%	1%	0	0	100%	Exp. 36
Two drops 1 : 5 : 7.5 c.c.	15			5%	1%	0	0	100%	Exp. 36
Five drops 1 : 5 : 7.5 c.c.	37.5			12%	2%	0	0	100%	Exp. 36
One drop 1 : 32 : 7.5 c.c.	1					0		85–90%	Exp. 14
Two drops 1 : 32 : 7.5 c.c.	2					0			Exp. 14
Four drops 1 : 32 : 7.5 c.c.	4					0			Exp. 14
Eight drops 1 : 32 : 7.5 c.c.	8					0			Exp. 14
Two drops 1 : 4 : 7.5 c.c.	16					0.2%		100%	Exp. 14
Four drops 1 : 4 : 7.5 c.c.	32					0.2%			Exp. 14
Eight drops 1 : 4 : 7.5 c.c.	64					0.5%			Exp. 14
Sixteen drops 1 : 4 : 7.5 c.c.	128					3%			Exp. 14

It will be noticed that inhibition is marked even at as low a concentration as one part of copper chloride in 2,500,000 parts of sea-water at a normal sperm concentration. Most of the experiments to be described were done with 1/500,000 copper chloride, at which concentration no eggs fertilize at normal sperm concentrations.

If much higher concentrations of sperm are used small percentages of eggs, varying somewhat in different experiments, may fertilize. (See last items of Table I.) There is thus a certain virtue in mass action of the sperm in the presence of this inhibitor of fertilization; this is somewhat difficult to understand, because only one spermatozoön penetrates usually. If we regard the spermatozoa, or some substance borne by them, and the copper as reacting with the same substance of the egg, it can be understood how the sperm substance could replace the copper in some cases when present in excess; or it may be possible that excess of sperm protects the eggs to a certain extent by combining with the copper and thus reducing the amount acting directly on the eggs.

2. *Reversibility of Copper Inhibition.*

Eggs that have been exposed to copper, whether in the presence of sperm or not, may be fertilized after return to sea-water provided that the exposure has not been long enough to injure their vitality too much. In other words, the inhibition by copper is reversible, and the phenomenon is to be regarded as one of inactivation of a substance, not of its destruction. In this respect, the phenomenon is precisely like the inactivation of an enzyme by mercury or copper salts, which is similarly reversible, and which has been shown to be due to a combining of the ions in question with constituents of the enzyme solution (v. Euler and Svanberg, 1920).

3. *Effect of Copper Chloride on Spermatozoa and Eggs Separately.*

A suspension of spermatozoa in 1/500,000 copper chloride has been tested up to 8 minutes exposure of the spermatozoa by fertilizing eggs in sea-water without any noticeable diminution in the fertilizing power of the sperm. The fertilizing power of a sperm suspension made in 1/25,000 copper chloride in sea-water began to fall off rapidly after two minutes; after four minutes only 5 per cent. of the eggs fertilized.

Eggs, on the other hand, show an effect of exposure to 1/500,000 copper chloride from 10 seconds exposure on, if transferred and fertilized in sea-water. The effect is seen first in poor viability, then in increase of polyspermy (after 1 minute exposure), but they may form membranes even after 8 minutes exposure. If exposed to 1/25,000 copper, 50 per cent. will not fertilize at all after 1 minute. The eggs in short are much more sensitive to the copper than the sperm.

Unfertilized eggs left in 1/500,000 copper chloride in sea-water begin to show visible signs of injury after about two hours at (approximately) 20° C. The surface of the egg beneath the membrane first appears roughened, then by degrees a perivitelline space appears containing a fluid stained red by escaping pigment; a true cytolysis involving a "laking" effect due to destruction of the plasma membrane has occurred. At this time, except for the presence of pigment in the perivitelline fluid, the eggs look as though they were provided with fertilization membranes. Following this, cytoplasmic buds appear, the nucleus swells and the egg disintegrates very gradually.

4. *Effect of Copper Chloride on Fertilized Eggs.*

(*a*) If eggs are fertilized in normal sea-water and transferred to 1/500,000 copper chloride in sea-water two or more minutes after insemination, they continue their development for several hours, up to a late cleavage stage at least; but the copper acts as a slow poison, so that the eggs rarely reach a swimming stage. Eggs may segment in 1/250,000 and 1/125,000 copper chloride if transferred 5 or more minutes after insemination in sea-water; but their rate of death is naturally increasingly rapid. In general the length of life of fertilized eggs is about the same in any concentration used, whatever be the time of transfer before cleavage. Successive stages of fertilization after the first five minutes do not appear to vary notably in their sensitiveness to $CuCl_2$ within the range explored. The effect of $CuCl_2$ on the stages of fertilization after the first few minutes is thus a progressive poisoning varying in rate with concentration.

There is thus an extraordinary contrast between fertilized and unfertilized eggs in their sensitiveness to copper; it is obvious that copper has an effect that belongs to a separate order of sensitiveness on the early stages of fertilization as compared with later stages.

(*b*) The question thus arises as to the time limits of the early inhibiting copper effect. A number of experiments were performed to test this point, two of which are presented in Tables II. and III. In these experiments the eggs were inseminated in sea-water and then transferred at intervals to the copper solution. Samples of the eggs shown in Table III. were transferred back to sea-water after fourteen minutes in the copper solution to test their viability.

TABLE II.

(EXP. 12.) EFFECTS OF COPPER CHLORIDE ON EGGS INSEMINATED IN SEA-WATER. A very heavy insemination was used in this experiment: two drops of 2 : 5 : 7.5 c.c.

Transfers to 1/500,000 $CuCl_2$.	Per Cent. Segmented.	Remarks.
(*a*) 4 seconds after insemination	10% +	Irregular cleavage.
(*b*) 6 seconds after insemination	10% +	Irregular cleavage.
(*c*) 10 seconds after insemination	80–90%	Irregular cleavage.
(*d*) 20 seconds after insemination	80–90%	Irregular cleavage.
(*e*) 30 seconds after insemination	100%	Irregular cleavage.
(*f*) 60 seconds after insemination	100%	Irregular cleavage.
(*g*) Control in sea-water	100%	Normal.
(*h*) Control inseminated in the Cu solution	1%	Irregular cleavage.

Here we must note two results particularly:

(1) Eggs that have begun fertilization reaction before transfer to the copper solution go through to cleavage, while those that have not begun it are instantaneously inhibited. Transfers made even four seconds after insemination (Table II.) include a considerable proportion of eggs (varying with sperm concentration in various experiments from 5 to 25 per cent.) which complete the fertilization and segment. The percentage rises to normal with increase of time before transfer.

(2) Eggs transferred from normal insemination to the copper chloride within the first two minutes do not give a complete

membrane reaction. The membranes of such eggs are "narrow," or better, the perivitelline space separating egg and membrane is narrow, often extremely so, down to the point of invisibility. The viability of such eggs is bad even if they are returned to sea-water again within a few minutes (Table III.). Indeed, the viability forms an ascending series to normal from 10 seconds up to 5 minutes. In other words, the fertilization reaction exhibits gradation in intensity according to the point of time at which the copper begins to operate, reaching normal at about two minutes with the best lots of eggs, and viability is proportional to fertilization intensity. There is no recovery from an initial subnormal reaction.

Table III.

(Exp. 37.) Effects of Copper Chloride on Eggs Inseminated in Sea-Water. Insemination, one drop of 1 : 5 : 5. Eggs replaced in sea-water after 14 minutes' exposure to $CuCl_2$. This experiment came at the end of the season when the eggs were not in first class condition.

	Transfers to 1/500,000 $CuCl_2$.	Per Cent. Segmented.	Remarks.
(*a*)	10 seconds after insemination	15%	Membranes "narrow." Viability bad.
(*b*)	20 seconds after insemination	30%	
(*c*)	25 seconds after insemination	30%	
(*d*)	40 seconds after insemination	50% +	
(*e*)	60 seconds after insemination	60%	Membranes "narrow." Viability better.
(*f*)	90 seconds after insemination	70%	
(*g*)	2 minutes after insemination	80%	Viability still better. Not equal to control.
(*h*)	5 minutes after insemination	80% +	Equal to control.
(*i*)	10 minutes after insemination	90%	

The period of time involved in these subnormal effects is that of membrane formation or of cortical discharge.

We have, thus, three distinguishable effects of copper chloride at successive stages of fertilization:

1. To inhibit activation if present at insemination.

2. To reduce the intensity of the cortical discharge, if its action begins within the first two minutes, proportionally to the time of its operation.

3. A slow cumulative injurious effect thereafter which belongs to a different order of events from 1 and 2, and which does not become obvious until late cleavage.

The data then give rise to the hypothesis that there is an activable substance present in the cortex of the unfertilized egg for which copper possesses special affinity. Copper thus prevents the inception of activation when present before insemination; during membrane formation it causes a quantitative reduction of activation. Thereafter, this substance having been consumed, the specific copper effects are absent.

Copper affects primarily the *activation* of this substance, and presumably not its *operation*, or at least to a very much less extent. The quantitative effects of the first two minutes can be understood in terms of the respective amounts activated and unactivated at the time of exposure to copper.

Using our earlier term *fertilizin* for the activable substance of the egg we may now attempt to form a picture of what actually happens in the copper chloride sea-water.

When a given lot of eggs is inseminated normally there is an interval of time taken up by the meeting of the spermatozoa and the eggs. The length of this interval will naturally vary statistically for any given insemination, and will tend to be inversely proportional on the average to sperm and egg concentration. Fertilization proper begins after the actual agglutination of the spermatozoön to the surface of the egg, following a latent period of variable duration. The activation of the fertilizin is then begun and the egg becomes sterile to other spermatozoa. As Just ('19) expresses it, a "wave of negativity" sweeps over the surface of the egg from the point of attack of the successful spermatozoön. Now the rate of this wave is sufficiently rapid to prevent polyspermy even at high sperm concentration where the eggs are in their best condition; the wave must therefore be completed very rapidly. As a result of activation the egg engulfs the spermatozoön within one minute or less, and the fertilization membrane elevates beginning at the point of entrance of the spermatozoön (cf. Just). Thus the events are (1) agglutination of the spermatozoön to the egg, (2) latent period, (3) activation and sterilization, (4) penetration of the spermatozoön, (5) membrane formation.

It does not necessarily follow that all of the fertilizin is activated in the first wave of negativity; this wave may be entirely superficial and activation may thence extend into deeper layers of the cortex. When, therefore, eggs are inseminated in sea-water and then transferred within the first few seconds to the copper sea-water there will be a certain proportion in which the initial reaction has begun, and this proportion will be larger according as the sperm concentration is higher, as actual experiments show. The copper will, however, instantaneously check the activation of more fertilizin so that the eggs must operate in the copper sea-water with that portion already activated. Transfers at later stages will be successively less affected by the copper, as successively greater amounts of the fertilizin will have been activated before transfer, until all is in operation. When this point is reached, at membrane elevation, the other events of fertilization proceed as well in the copper sea-water as in normal sea-water.

If this form of interpretation is correct, we can perhaps see a little farther into the fertilization reaction. If activation of fertilizin follows immediately after agglutination of the spermatozoön to the egg, we would expect in the case of a heavy insemination that activation would have begun in all of the eggs within a few seconds,, say 5 to 10, after insemination, because it is practically certain that in that space of time all eggs will have agglutinated spermatozoa. However, we notice in both of the tables and especially in Table III. that the proportion of eggs that segment does not suddenly rise to normal in a ten-second exposure. In some cases the rise is relatively sudden, as in Table II., in other cases relatively slow, as in Table III., and this corresponds to the physiological condition of the eggs as shown by the promptness of their behavior in normal fertilization. In other words, it is necessary to postulate a latent period after agglutination of the spermatozoön before activation begins, which varies with the physiological condition of the egg. This fact could be brought out only by use of a reagent like copper that instantaneously checks activation. When the wave of activation is begun it spreads flash-like over the surface, though this rate varies also with physiological condition as shown by the relation of the latter to polyspermy. It is not necessarily the first spermatozoön that agglutinates that effects the initial discharge, but this will depend on physiological condition of the spermatozoön, and also perhaps on local differences of physiological condition on the surface of the egg.

5. *The Effect of Copper on Agglutination of Spermatozoa.*

The objection might be raised that copper inhibits by preventing agglutination of the spermatozoön to the egg, and not in the later activation of the fertilizin. The effect of copper on the agglutination reaction of the spermatozoa was therefore studied: Egg-water (of 1,600 agglutinating units) diluted 100 times or more with 1/500,000 copper chloride in sea-water has the same agglutinating effect on a sperm suspension in the same copper solution as when no copper is present either in the egg-water or sperm suspension. Agglutination will occur even in the presence of one part of copper chloride to 25,000 parts of sea-water, though more slowly. Copper chloride in the concentrations employed in the fertilization experiments has no noticeable effect on rate or duration of the agglutinating reaction.

The inhibiting action of copper must then occur after the agglutination of the spermatozoön to the egg, as was assumed in the preceding discussion.

6. *Protective Action of Egg-Water Against Copper.*

If copper chloride inhibits by combining with the fertilizin of the egg, then egg-water which contains fertilizin should protect against the inhibition of fertilization by deviating the copper from the fertilizin in the egg to that in the egg-water. Now it is known that egg-water contains a sperm-activating, a sperm-aggregating and a sperm-agglutinating substance (Lillie, '14, '19), and I have identified the sperm-agglutinating substance with fertilizin. These considerations led to experiments on the protective action of egg-water on the copper inhibition, in which definite positive results were obtained.

The egg-water is obtained by placing eggs in sea-water which receives their secretions. The strength of the egg-water depends on egg-concentration primarily, and also on time to a certain extent; it may be measured, as far as the sperm-agglutinating constituent of the egg-water is concerned, by finding the greatest dilution at which it will cause visible agglutination in a sperm suspension. If the proportion of eggs to sea-water is about 1 to 4 in bulk, the egg-water from fresh eggs will usually stand dilution to about 1/1,600, and would therefore be accordingly rated as 1,600 units agglutinating strength. Other constituents of the egg-water are diluted at the same time naturally, but there is no present means of measuring these.

In a preliminary experiment (No. 21) eggs were identically inseminated (one drop of 1:25:7.5 c.c.) in (*a*) normal sea-water, (*b*) in sea-water to which one part of copper chloride in 500,000 parts of sea-water had been added, (*c*) in egg-water of 1,600 agglutinating units to which also one part of copper chloride in 500,000 parts of egg-water had been added. In (*a*) 100 per cent. of the eggs segmented, in (*b*) none segmented, in (*c*) 68.5 per cent. segmented. The egg-water was thus shown to protect against the copper chloride.

More elaborate experiments were then set up, one of which is given in Table IV. Here it is shown that egg-water alone reduces the percentage of cleavage from 80 per cent. (i, control 1) to 50 per cent. (k, control 3); that 1/500,000 copper chloride in sea-water completely inhibits fertilization (j, control 2), and that egg-water protects completely against this concentration of copper chloride down to 80 units (a, b, and c), and that its protective action at lesser concentrations falls off to zero (d, e, f, g, h).

TABLE IV.

(Exp. 23.2.) Identical Inseminations in the Fluids (*a*) to (*k*). (One drop of 1 : 25 : 7.5 c.c.)

		Per Cent. of Eggs Segmented.
(*a*)	1/500,000 Copper egg-water of 400 agglutinating units	50%
(*b*)	1/500,000 Copper egg-water of 200 agglutinating units	50%
(*c*)	1/500,000 Copper egg-water of 80 agglutinating units	50%
(*d*)	1/500,000 Copper egg-water of 40 agglutinating units	15%
(*e*)	1/500,000 Copper egg-water of 20 agglutinating units	10%
(*f*)	1/500,000 Copper egg-water of 10 agglutinating units	<0.1%
(*g*)	1/500,000 Copper egg-water of 7 agglutinating units	<0.1%
(*h*)	1/500,000 Copper egg-water of 5 agglutinating units	0
(*i*)	Sea-water alone. Control 1	80%
(*j*)	1/500,000 Copper sea-water. Control 2	0
(*k*)	Egg-water alone (400 units). Control 3	50%

7. *Effects of H and OH Ions on Copper Inhibition.*

Two series of experiments (Nos. 24 and 25) were run to see if increase in H or OH ions protected against the inhibitory action of copper chloride. As might have been expected, increase in acidity of the sea-water had no favorable effect. On the alkaline side some protective action is found between about 1/2,500 N NaOH and 1/666 N NaOH by volume. In the best case about 33 per cent. of the eggs segmented when 1/666 N NaOH was added to 1/500,000 copper chloride sea-water. The membranes were, however, very narrow, and the cells tended to separate in cleavage owing to this fact. The appearance of the eggs was similar to eggs segmenting in a copper solution too weak to produce complete inhibition. The effect was no doubt due to precipitation of some of the $CuCl_2$ as $Cu(OH)_2$, but the dissociation of the copper salts was still sufficient to inhibit very greatly.

8. *The Protective Action of Gum Arabic and Gelatin Against Copper.*

Egg-water stands by no means alone in protecting against the inhibiting action of copper on fertilization. The same result may be obtained with either gum arabic or gelatin, and presumably with other colloids and proteins.

The protective action of gum arabic begins at 0.2 per cent. and becomes complete at 0.8 per cent. (see Table V.). As the gum arabic has no deleterious action of its own it furnishes complete protection at the proper concentration, as the table shows.

Table V.

(Exp. 32.) Protective Action of Gum Arabic. Identical inseminations in the fluids (*a*) to (*e*) and their controls. (One drop of 1 : 25 : 7.5 c.c.)

One Part of $CuCl_2$ to 500,000 Parts of	Per Cent. of Eggs Segmented.	Same Solutions Without $CuCl_2$ (Controls).	Per Cent. Segmented.
(*a*) 0.2% gum arabic in s.w.	10%	(*a*) 0.2% gum arabic in s.w.	100%
(*b*) 0.4% gum arabic in s.w.	55%	(*b*) 0.4% gum arabic in s.w.	100%
(*c*) 0.8% gum arabic in s.w.	100%	(*c*) 0.8% gum arabic in s.w.	100%
(*d*) 1.6% gum arabic in s.w.	100%	(*d*) 1.6% gum arabic in s.w.	100%
(*e*) Sea-water (copper control)	0	Sea-water only	100%

The protective action of gelatin begins at 0.008 per cent. and becomes complete at 0.064 per cent. (see Table VI.).

TABLE VI.

PROTECTIVE ACTION OF GELATIN (EXP. 34). Identical inseminations in the fluids (*a*) to (*j*) and their controls. (One drop of 1 : 25 : 7.5 c.c.)

One Part of $CuCl_2$ to 500,000 Parts of	Per Cent. Segmented.	Same Solutions Without Copper (Control).	Per Cent. Segmented.
(*a*) Sea-water (control)	0	(*a*) Sea-water (control)	100%
(*b*) 0.0001% gelatin in s.w.	0	(*b*) 0.0001% gelatin in s.w.	90%
(*c*) 0.001% gelatin in s.w.	0	(*c*) 0.001% gelatin in s.w.	90%
(*d*) 0.002% gelatin in s.w.	0	(*d*) 0.002% gelatin in s.w.	100%
(*e*) 0.004% gelatin in s.w.	<1%	(*e*) 0.004% gelatin in s.w.	100%
(*f*) 0.008% gelatin in s.w.	20%	(*f*) 0.008% gelatin in s.w.	100%
(*g*) 0.016% gelatin in s.w.	50%	(*g*) 0.016% gelatin in s.w.	100%
(*h*) 0.032% gelatin in s.w.	90%+	(*h*) 0.032% gelatin in s.w.	100%
(*i*) 0.064% gelatin in s.w.	100%	(*i*) 0.064% gelatin in s.w.	100%
(*j*) 0.1% gelatin in s.w.	100%	(*j*) 0.1% gelatin in s.w.	100%

Thus both gum arabic and gelatin protect against the inhibiting effect of copper. Presumably any non-injurious substance that would form a non-dissociable compound with copper, thus removing it from the sphere of action of fertilization, would similarly protect. The question then arises whether the protective action of egg-water is a special instance of the general colloid (gum arabic) effect, or of the protein (gelatin) effect. I think we may say definitely that the action of egg-water is not a general protein effect, for even the strongest egg-water does not give any certain protein reaction. It is moreover practically certain that diluted egg-water which protects does not have a colloid content equivalent to the protective minimum of the gum arabic solution. There is something in the egg-water that does not give protein reaction, but with probably an equal or greater avidity for copper. There is also something in the cortex of the egg itself with a similar affinity for copper, viz.: the activable substance. It is reasonable to suppose that these two things are identical.

It is an interesting fact that egg-water also possesses a deviating effect on the inhibiting action of species-blood in fertilization which is not a general colloid effect nor yet a general protein effect (Lillie, 1914). As egg-water protects against two such different forms of inhibitor, it is reasonable to suppose it is able to do so by possessing the same substance as that on which the inhibitors work in the egg. No other hypothesis possesses the simplicity of this one, which means that the activating substance of the egg (fertilizin) is present in the egg-water.

9. *The Effect of Copper on Activation by Butyric Acid.*

If copper chloride inhibits fertilization by combining with the fertilizin of the egg it should also inhibit activation of the egg by parthenogenetic agents as, *e.g.*, butyric acid. This consequence of the theory was found to be true. If unfertilized eggs of *Arbacia* are placed in 50 c.c. sea-water + 2 c.c. $N/10$ butyric acid, and transfers are made to 1/500,000 copper sea-water and to sea-water for control, after 30 seconds and 45 seconds, it is found that in the sea-water good to fair membranes form on practically all of the eggs in about two minutes; but in the 1/500,000 copper sea-water no membranes form and the eggs appear entirely unchanged for at least twenty minutes. Then a gradual cytolysis begins to set in, entirely similar to the cytolysis that occurs in copper sea-water without previous exposure to butyric acid after two to three hours. The butyric acid has hastened the appearance of the copper cytolysis; but the copper has entirely inhibited the typical membrane-forming reaction.

Copper present with the butyric acid does not, however, inhibit the membrane formation after transfer to sea-water. In this reciprocal experiment one part of copper chloride is added to 500,000 parts of the butyric acid solution, and the eggs receive the normal exposure. When transferred to sea-water, membranes form just as though the copper had not been present. Thus, whatever the pre-activation effect of the butyric acid may be, copper does not inhibit it, but operates only to prevent the activation of the fertilizin.

Similarly in fertilization the preliminary effect of the spermatozoön in preparing the fertilizin for discharge ("latent period"; see p. 141) is not presumed to be affected by the copper but only the actual discharge (*i.e.*, activation) of the fertilizin.

10. *A Comparison of the Effects of Mercuric Chloride.*

It will be desirable to compare the effects of the salts of other heavy metals on fertilization, and some experiments have been begun along this line. However, in this paper, mercury alone will be considered.

The effect of mercuric chloride ($HgCl_2$) on fertilization is pronounced, but it is very different from that of copper chloride: the initial stages of fertilization are relatively little affected, and the susceptibility increases as fertilization progresses; fertilized eggs show the effects much more rapidly than unfertilized. Mercury also suppresses the movements of the spermatozoa at great dilution, and offers in this way another contrast to copper.

Table VII. records an experiment in which eggs were inseminated in various dilutions of $HgCl_2$ in sea-water, between one part in 1,250,000 and one part in 15,625. Most of the eggs form fertilization membranes in the four lowest concentrations. But the eggs do not segment, except at the lowest concentration tested, and then only 20 per cent. irregularly. The fertilized eggs cytolyze more rapidly than the unfertilized.

Table VII

Experiment 31A. Eggs Inseminated in Series of $HgCl_2$ Solutions in Sea-water. (One drop of 1 : 25 : 7.5 c.c.)

$HgCl_2$ in Sea-water.	Membranes.	Cleavage.	Cytolysis Begins.	Action on Sperm.
1. 1/1,250,000 . . .	100% good	20% irreg.		Faint movement only in 3 min.
2. 1/625,000.	100% good	None		Same
3. 1/312,500.	90%+ good	None	68 minutes	Same
4. 1/156,250.	90%+ good	None	Bad in 1 hour	Paralyzed, 3 min.
5. 1/125,000.	20% good 80% none	None	Fert. eggs cytolyze in 20 minutes	Paralyzed in few seconds
6. 1/62,500.	15% good 85% none	None	Same as above	
7. 1/31,250.	2% 98% none	None		
8. 1/15,625.	None	None		Paralyzed instantly
9. Sea-water control.	100% good	100%		

It was also observed that unfertilized eggs form membranes in solution 5 (Table VII.) beginning at about five minutes. When transferred to sea-water such eggs undergo changes similar to eggs treated with butyric acid.

Mercury thus exhibits an extraordinary contrast to copper: in certain comparable concentrations it paralyzes sperm, copper does not; it produces membrane formation alone, and favors it in fertilization, copper inhibits membrane formation; it suppresses cleavage and cytolyzes fertilized eggs rapidly; copper is neutral during the same stages.

The differences in the actions of copper and mercury respectively on fertilization may be explained by differences in intensity of action on the early and the later stages of fertilization respectively. In the case of mercuric chloride I have found that the inhibiting action in fertilization (which occurs at higher concentrations than in the case of copper, as for instance in solutions 5 and 6 of Table VII.) is reversible, like copper. Specifically, eggs that remain unfertilized in these solutions may be fertilized after return to sea-water, if the exposure is not too long (about 20 minutes). Presumably, therefore, mercury acts like copper in the initial stages of fertilization, though a higher concentration is required; but in the later stages mercury acts deleteriously in much lower concentrations than copper; one part of $HgCl_2$ in 625,000 parts of sea-water will completely inhibit cleavage of fertilized eggs, whereas it requires about one part of $CuCl_2$ in 62,500 parts of sea-water to produce comparable effects.

The comparison may be tabulated as follows according to the stages of fertilization:

1. Agglutination of the spermatozoön to the egg.
2. Latent period.
   Copper intervenes, Hg not, or Cu > Hg.
3. Combination of sperm receptors with fertilizin.
4. Activation = combination of fertilizin with egg receptors.
5. Sterilization = "wave of negativity."
6. Spermatozoön enters the egg.
7. Membrane formation.
8. *Hg > Cu in later events.*

It is well known that the salts of heavy metals have a powerful "poisonous" effect on enzymes. McGuigan (1904) determined that for diastase the order of poisonous effects (complete inhibition) is *N*/100,000 silver (nitrate), *N*/33,333 gold (chloride), *N*/30,000 mercury (chloride), *N*/8,333 copper (chloride). v. Euler and Svanberg (1920) studied the effects of heavy metals on the inversion of cane sugar by saccharase, and determined among many other things that mercury has about one thousand times the poisonous effect of copper; the metallic ions in question enter into combination with some constituent of the enzyme solution.

The order of poisonous effects of mercury and copper on the events of fertilization following membrane formation is thus the same as that of the poisonous action of these metals on enzymes. The concentrations also are of comparable magnitude (cf. McGuigan's data above). v. Euler and Svanberg found the range for $HgCl_2$ between complete inhibition of the ferment and no effect to lie between 1 part in 116,600 and 1 part in 1,166,000 of the solution (compare Table VII. of this paper). There is thus a rather surprisingly close agreement in the effective concentrations; so that it is reasonable to conclude that the effect of mercury and copper on fertilization following membrane formation may be due to enzyme poisoning.

The inhibiting effect of mercury and copper on the initial stages of the fertilization reaction is in the inverse order, and does not, therefore, correspond so well to the enzyme analogy. The results emphasize the strong contrast between the initial and the subsequent events of the fertilization process, which is found also in the phenomena of specificity (Lillie, 1919, 1921), and in other phenomena of fertilization.

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PHAGOCYTOSIS AND CLOTTING IN THE PERIVISCERAL FLUID OF ARBACIA.

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The perivisceral fluid of the Echinoidea contains certain formed cellular elements which have been observed and described by Valentin ('42), Williams ('56), Hoffman ('72), Prouho ('86), Geddes ('80), Cuénot ('91) and Théel ('96). For most of our knowledge of these cellular elements we are indebted to Geddes and Cuénot. Goodrich ('19) in his discussion of the leucocytes of the invertebrates is the most recent contributor.

Cuénot observed that these formed elements were found not only in the perivisceral cavity, but also in the lumina of the ambulacral system; in the lacunæ of the intestinal walls and in all other organs of the body. He further observed that they were not all of one type, but could be divided into two distinct series, each of which series could be further subdivided. The first series of cells he called "vibratile globules," minute cells 6–9 micra in diameter having a head and vibratile tail. The head is mostly nucleus enclosed in a thin sheath of cytoplasm. According to Cuénot the function of this type of cell is to keep the fluid contents of the perivisceral cavity in motion.

The second series of cells were called "amibocytes" and were subdivided into several different types. The first type, from which Cuénot assumed the other types to be derived, was found abundantly in the lymph-glands. The cells of this type were characterized by a homogeneous cytoplasm, long and numerous pseudopodia, and a relatively large nucleus within which was a distinct nucleolus. Sometimes the amibocytes of this type enclosed yellow or brown refractile granules. According to Geddes these were the most numerous of the corpuscles and all of the investigators have agreed that these cells are the chief agents in the formation of the blood clot. Because of this activity

Dekhuyzen ('01) named them "thrombocytes" and compared them to the blood platelets of the vertebrates. Cuénot, Geddes and Dekhuyzen believed that when a clot was formed, these cells fused with each other to form larger true plasmodial masses, the cells of such a mass losing their identity. In order to check this assumption, Geddes introduced colored granules into the perivisceral fluid and observed that these colored granules were to be found in the large multinucleate masses which were formed by the fusion of the amibocytes. Michel ('88) and Goodrich ('19) claim that these cells do not form true plasmodial masses, but do form aggregate plasmodia in which the cells retain their identity and are in contact by their ectoplasmic surfaces alone. Goodrich states further that these cells, instead of having a number of delicate pseudopodia, are under normal conditions provided with numerous flap-like membranous processes similar to the undulating membranes of the Protozoa. These membranous flaps when viewed in optical section have the appearance of delicate pseudopodia. The membranous flaps are constantly changing and when a drop of the perivisceral fluid is taken out of the cavity and spread out on a slide, these cells clump together if approximate to each other; if isolated, the membranes shrink as the fluid evaporates leaving long, filamentous strands of ectoplasm.

The other types of amibocytes are larger than those just described and contain colored or colorless globules. They also have a relatively smaller nucleus and form blunt pseudopodia when moving in the perivisceral fluid. Various functions have been ascribed to these larger amibocytes. The red, green and yellow material appearing in these amibocytes was said by Cuénot to be stored fat; by McMunn to be a respiratory pigment which he called by the non-committal name of echinochrome; and by Ribaucourt this colored material was thought to be excretory matter which was to be carried out of the body through the ambulacral system by these amibocytes. They were all agreed that these amibocytes were derived from the first type of amibocyte by the inclusion of this material or by its precipitation after absorption. Since these nutritional or nephritic cells take but a passive part in the formation of a clot and none at all in phagocytosis, they will not be considered further. The name leucocyte which Goodrich applied to the first type of amibocyte will be used here to designate the type of amibocyte active in the process of phagocytosis and clotting.

The leucocytes of *Arbacia* have the same general appearance as those figured by Goodrich for *Asterias*, but the membranes of the leucocytes of *Arbacia* seem to be larger in comparison to the amount of endoplasm than do those of *Asterias.* As the leucocyte moves about in the plasma medium the membranes are constantly changing (Fig. 1, *a*, *b*, *c*). In the living condition, the cytoplasm of the leucocyte appears finely granulate and contains five or six hyaline vacuoles. The nucleus is scarcely discernible in the living cell, but when treated with Flemming's or Herman's fluid it appears as a large ovoid body with a very distinct membrane. The chromatin granules are very prominent in the nucleus of those cells stained with Haidenhain's

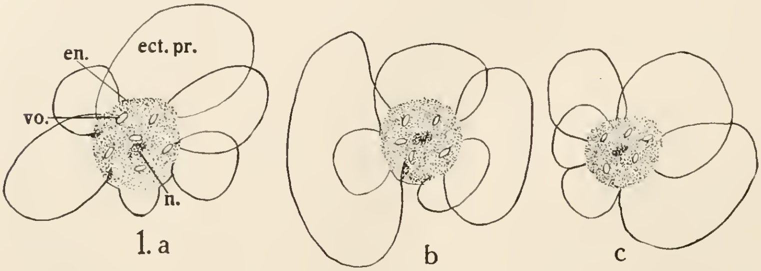

FIG. 1, *a*, *b*, *c*. Leucocyte of *Arbacia* moving freely in plasma medium, drawn at five-minute intervals. Semi-diagrammatic. × 1,500. *ect. pr.*, ectoplasmic process; *en.*, endoplasm; *n.*, nucleus; *vo.*, vacuoles.

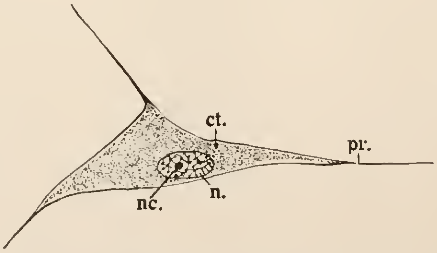

FIG. 2. Leucocyte of *Arbacia* from clot. Semi-diagrammatic. × 2,500. *ct.*, cytoplasm; *n.*, nucleus; *nc.*, nucleolus; *pr.*, ectoplasmic process.

hæmatoxylin. A large nucleolus is also present. When fixed with Schaudinn's fluid and stained with Haidenhain's hæmatoxylin, the cytoplasm appears reticulate (Fig. 2).

I. PHAGOCYTOSIS.

Carmine and india ink suspensions in sea-water were used in order to determine the extent to which the leucocytes took part in the inclusion of foreign particles from the perivisceral fluid. One cubic centimeter of sea-water suspension of finely powdered carmine was introduced into the perivisceral fluid through a perforation in the peristomial membrane. The individual so treated was put back into the aquarium and at the end of thirty minutes a drop of the perivisceral fluid was examined. Although a large number of carmine grains were yet free in the plasma, it was observed that all of the leucocytes had taken up one or more particles. At the end of an hour another examination was made of the perivisceral fluid and at this time the plasma was comparatively free from carmine particles and the leucocytes were heavily loaded with them. The ingestion of several of these particles by the leucocyte was observed. This individual was kept in the aquarium for two weeks during which time the perivisceral fluid was examined at intervals. At the end of two weeks some leucocytes containing carmine particles were still present in the perivisceral fluid. This fact indicates a period of longevity on the part of these cells, but the fate of the large number which performed the function of phagocytes is still in doubt. Ribaucourt suggested that after they had become loaded with waste material from the perivisceral fluid they made their way into the ambulacral system by diapedesis and thence passed out through the madrepore. Since, however, no currents have been observed nor any cells observed leaving the madrepore, this assumption is unconvincing. The problem of excretion and with it the fate of the old phagocytes has yet to be solved.

The results obtained by the injection of 1 c.c. of india ink-sea-water suspension were the same as those obtained with the carmine suspension. And further in the study of the clot, it was observed that the ingestion of particles by the leucocytes does not inhibit their activity in the formation of the clot.

In both of these experiments the amibocytes other than phagocytes did not act as phagocytes. This may be due to the fact that, if they are, as assumed, derived from leucocytes in the first place by the inclusion of reserve food material or absorption of nitrogenous waste, the material so added inhibits any phagocytic activity.

II. Reactions to Vital Stains.

No success was met with in the attempt to stain the leucocytes by the injection of weak intravitam stains. Aqueous solutions of methyl blue, methyl green, Janus green, and trypan blue were introduced into the perivisceral cavity, but no effect on the formed elements was noted.) A 1:500 methyl blue-sodium chloride solution had no effect upon the leucocytes, but faintly stained the globules in the red amibocytes ("globules amœboide brun d'acajou" of Erdle).

One c.c. of .01 per cent. neutral red in sea-water was injected into the perivisceral fluid of several individuals and after twenty-four hours all of the leucocytes in several drops of the perivisceral fluid examined contained yellow globules. This indicated that the hyaline globules apparent in the leucocyte under normal conditions have an alkaline content.

III. Clotting.

A drop of the perivisceral fluid of *Arbacia*, freshly drawn, when placed on a cover slip immediately shows signs of agglutination. The large pigmented and colorless amibocytes appear in long strings held together by the clumping of the leucocytes, although in many cases the leucocytes had not lost their membranous processes. A drop of the perivisceral fluid was placed on a glass slide and observed continually for two hours. Sketches of the changes which were taking place in the shape of the cells in a restricted area were made at intervals of thirty minutes in order to show the rôle played by the leucocytes in the formation of the clot. When first observed there were four leucocytes in the field, each possessing the typical membranous projections (Fig. 3). As the fluid evaporated during the course of an hour these leucocytes came into contact with the slide.

Whereupon the membranous processes of the ectoplasm began to shrink and the leucocyte to elongate (Fig. 4). Several red, yellow and colorless amibocytes which were in the vicinity adhered to the margins of the leucocytes as if the latter were secreting a sticky substance. At the end of one and a half hours the leucocytes had become extremely elongate and the membranous projections had entirely disappeared (Fig. 5). The filamentous projections left from the membranes extended across the field and came into contact with those of the neighboring leucocytes so that a meshwork was established to which the larger pigmented and colorless amibocytes adhered..

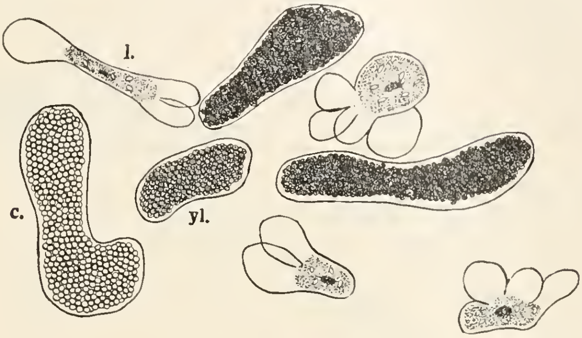

FIG. 3. Group of cells in perivisceral fluid on slide. Semi-diagrammatic. × 1,000.

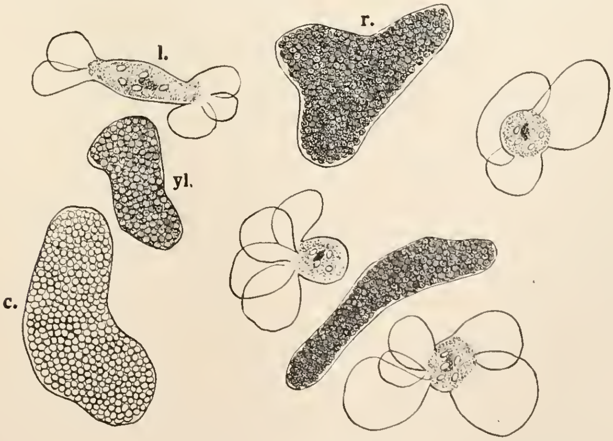

FIG. 4. Same group of cells as in Fig. 3, drawn 30 minutes later to illustrate beginning of clot formation. Semi-diagrammatic. × 1,000.

The activity of the leucocyte was then observed in a hanging drop, but typical clot formation did not occur except where the leucocytes came in contact with the glass. If the leucocytes in this drop were isolated from each other they retained their membranous projections, but if they came into contact with each other they tended to stick together and form a mass to which the other amibocytes adhered. A meshwork such as observed under the conditions of evaporation did not appear. I was unable however to observe whether or not the leucocytes formed true plasmodia or only aggregate plasmodia. If their reaction to each other in forming these masses is similar to the manner in which they form the meshwork of the clot then they must certainly retain their individuality and come into contact without cytoplasmic fusion.

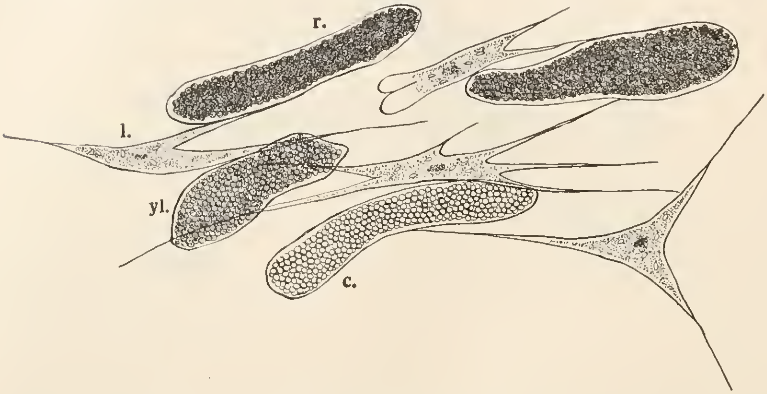

Fig. 5. Same group of cells as in Figs. 3 and 4. Clot fully formed. Drawn 30 minutes later than Fig. 4. Semi-diagrammatic. × 1,000. *c.*, amibocytes with colorless globules; *l.*, leucocyte; *r.*, amibocyte with red globules; *yl.*, amibocyte with yellow globules.

An attempt was made to find out the rôle the leucocyte played in the regeneration of resected parts. In the case of a perforation or a slit in the peristomial membrane, the leucocytes form a clot along the margins of the wound. This clot gradually extends across the opening and eventually closes it. If a piece of the test is removed, the leucocytes form a clot around the margin of the perforation and after a longer or shorter period depending upon the size of the piece cut out, this clot closes it. The further history of the relation of the clot to the tissues originally occupying the resected area will be followed later.

SUMMARY.

1. Phagocytosis in the perivisceral fluid of *Arbacia* is carried on by the leucocytes alone. These cells possess membranous processes similar to those of the leucocytes of *Asterias* and are the smallest and most numerous of the amibocytes. As phagocytes the leucocytes have a long period of activity.

2. The leucocytes tend to agglutinate even in freshly drawn perivisceral fluid. In a drop of perivisceral fluid on a plane surface, the leucocytes lose their characteristic membranous processes upon contact with the surface and become elongate. Long filamentous processes are produced by the shrinkage of the membranous processes. These filamentous processes adhere to those of neighboring leucocytes forming a delicate mesh to which the pigmented and colorless amibocytes adhere.

3. The leucocytes show an alkaline reaction when treated with neutral red in sea-water, but are not affected by weak solutions of intravitam stains introduced into the perivisceral cavity.

4. If a piece of the peristomial membrane or the test is resected, the leucocytes form a clot which closes the wound.

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STUDIES ON THE PARASITIC WASP, HADROBRACON BREVICORNIS (WESMAEL).

II. A Lethal Factor Linked with Orange.

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Linkage of genetic factors has been demonstrated in many widely diverse species of plants and animals. In animals it may be complete in either sex, partial in the other; or it may be partial in both sexes. In plants it is apparently partial in both types of sporogenesis. Up to the present time linkage has not been demonstrated in Hymenoptera. In species which produce males by haploid parthenogenesis we might expect linkage in ovogenesis to be partial. In spermatogenesis linked factors would, of course, be completely linked as in the case of sex-linked factors in *Drosophila*. Unlike sex-linkage, however, there would be several independently segregating sex-linkoid groups, corresponding to the reduced number of chromosomes.

In the parasitic wasp, *Hadrobracon*, orange eye color is inherited as a sex-linkoid recessive to typical black. Heterozygous females produce black and orange males in equal numbers as previously shown.[1]

Three heterozygous sisters were isolated in July, 1920. One, a virgin, produced 38 black males and 44 orange males.

The second was bred as a virgin and later mated to one of her orange-eyed impaternate sons so that females were produced. Her progeny consisted of 57 black males, 44 orange males, 37 black females and 29 orange females. One of her black daughters isolated as a virgin produced 14 black males and 16 orange males.

Under normal conditions full-grown larvæ of the wasp spin cocoons before pupating which serve to attach them firmly in the culture vial. If the supply of food is insufficient some of the larvæ pupate without spinning cocoons and are shaken into the ether bottle when the first count is made. If returned to the vial they will, in most cases, mature at the expected time.

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[1] Whiting, P. W., "Studies on the parasitic wasp, *Hadrobracon brevicornis* (Wesmael)—I., Genetics of an Orange-eyed Mutation and the Production of Mosaic Males from Fertilized Eggs," Biological Bulletin, Vol. XLI., No. 1.

The progenies of the heterozygous females above recorded consisted of black males, 104 adults, 5 pupæ; orange males, 101 adults, 3 pupæ; black females, 37 adults, no pupæ; orange females, 28 adults, 1 pupa. The ratio is quite in agreement with expectation and nothing unusual was noticed about the pupæ. Some of them would probably have metamorphosed to adults had they been replaced in the culture vial.

The third heterozygous black sister isolated as a virgin-produced offspring in an unexpected ratio. It was first noticed that the adult progeny were almost all orange and that there was an unusual number of small-sized pupæ without cocoons. Examination of the latter, moreover, showed that most of them had black eyes. It was immediately suspected that there was a lethal factor coupled with the normal allelomorph to orange. Consequently all pupæ were kept beyond the normal time of eclosion and those that failed to mature were counted as lethal. The black-eyed mother was mated to an orange son in an attempt to get black-eyed daughters carrying the lethal. The total progeny of this lethal-bearing female consisted of black males, 4 adults, 28 pupæ; orange males, 44 adults, 4 pupæ; black females, 2 adults, 3 pupæ; orange females, 18 adults, no pupæ. Among the females the excess of orange over black adults and the presence of black pupæ might seem to indicate partial dominance of the lethal factor. In any case the males show a striking departure from expectation. Black pupæ and orange adults would be *straights* and black adults and orange pupæ would be *crossovers*, except that a few of the pupæ might have failed to eclose on account of partial starvation. Among the males of this one fraternity there were 8 crossovers and 72 straights or 10 per cent. crossovers.

The two black-eyed daughters were isolated and later bred to orange sons. One produced black males, 5 adults, 17 pupæ; orange males, 23 adults, 6 pupæ; black females, 2 adults, 2 pupæ; orange females, 2 adults, no pupæ.

The other produced black males, 11 adults, 27 pupæ; orange males, 30 adults, 11 pupæ; black females, 3 adults, 1 pupa; orange females, 3 adults, 2 pupæ.

The lethal factor is therefore inherited, there being in this generation 33 crossovers and 97 straights or 25 + per cent. crossovers.

Although considerable care was taken to preserve the lethal stock it very rapidly died out due to difficulty in getting black-eyed lethal-bearing females, and moreover the total numbers in the fraternities became very small. Only three black-eyed females had offspring in the next generation and these were all males. One produced black, 5 adults, 27 pupæ; orange, 20 adults, 6 pupæ.

A second produced black, 3 adults, 12 pupae; orange, 15 adults, 4 pupæ.

The third produced all adults, 5 black and 9 orange. Although the numbers are very small in this last-mentioned fraternity the failure of any lethal pupæ to appear in spite of the presence of 5 adult black males is good evidence that the mother originated from a crossover or non-lethal black-bearing egg.

The total male progeny of all five lethal-bearing females is black, 28 adults, 111 pupæ; orange, 132 adults, 31 pupæ; 59 crossovers to 243 straights, or 19.5 per cent. crossovers.

Partial linkage is therefore demonstrated in Hymenoptera.

During the experiments above recorded a number of the lethal pupæ were fixed in Carnoy's fluid for histological examination. Sagittal sections, stained with iron hæmatoxylin, showed no abnormal growths or defects in any organs. The nature of the lethal effect is therefore unknown.

EXPERIMENTS ON THE DEVELOPMENT OF THE GILLS IN THE AMPHIBIAN EMBRYO.[1]

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The present work upon the gills was undertaken as a continuation of the study of relations of symmetry in embryonic organs, in the hope that it might throw light on questions raised in previous studies of the limbs.[2] The external gills of the salamander were chosen to this end, as constituting another system with right and left enantiomorphs, each without any plane, axis, or center of symmetry in itself. Owing to the complexity of the system, however, if for no other reason, the experiments failed to give as clear evidence on the questions raised as the limbs had done. Nevertheless, they have brought to light certain interesting facts, which in part corroborate and in part extend those reported by Ekman ('13), who worked upon various anuran embryos. They show, furthermore, certain differences between *Amblystoma* and the anurans with respect to the organs studied.

The gill arches and external gills form a complex in which all of the germ layers are represented. There is an outer covering of ectoderm, a central core of mesoderm, which contains the blood vessels and extends out into the gill filaments, and an inner lining to the arches and the clefts consisting of endoderm. Besides these the ganglion crest contributes elements to the arches, chiefly to the formation of the cartilaginous skeleton.

The experimental work of Ekman upon anurans has shown the following: (1) That when the ectoderm covering the gill region is removed, the regenerated ectoderm is capable of forming gill filaments. (2) That when the gill ectoderm is replaced by ectoderm from another region, with certain exceptions, the formation of gills is suppressed. (3) That the external gills and the outer branchial grooves are specifically determined in the ectoderm and that the endoderm plays no part in the localization of the gills. (4) That the ectodermal rudiments of the gills have, from early stages on, a certain power of self-differentiation which varies amongst the several species studied. (5) That gills which arise from displaced ectoderm, together with atypical endoderm, conform entirely as to their position, form, and size, to the ectoderm. (6) That the branchial blood vessels are an essential factor in the later development of the gills.

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[1] Read before the National Academy of Sciences, November 17, 1920

[2] Harrison ('17 and '21).

The present experiments were made upon the embryo of a urodele, *Amblystoma punctatum*. They concern almost entirely the two outer layers, ectoderm and mesoderm. Owing to technical difficulties, the endoderm was experimented with only to a very limited extent, and this layer, therefore, does not figure in the present analysis. While certain things are quite definite, the work must be considered as incomplete, inasmuch as there has been no opportunity as yet to examine the specimens in sections.

With regard to the technique of operation, only one thing requires special mention. The donor embryo was in most cases stained *intra vitam* in Nile blue sulphate, which colors the ectoderm bright blue.[3] The stain persists for many days, so that the exact delimitation of the graft can be observed, as development proceeds. This removes a serious source of error, for it is thus always possible to determine whether the parts under observation are derived from the host or the graft. Embryos from stage 21 (Fig. 1, medullary folds just closed) to stage 25 (just before appearance of tail bud[4]) were used for nearly all the experiments. A few were made with earlier and a few with slightly later stages. Subsequent experiments made with still later stages show that marked changes in the behavior of the ectoderm after transplantation occur at about the time when the mesectoderm from the ganglion crest grows down and the first branchial pouch is developed (stage 29). These will not be considered at present.

[3] Detwiler ('17).

[4] Stages 25 and 29 have been figured previously (Harrison '18, Figs. 1 and 2).

Removal of the Ectoderm of the Branchial Region and its Replacement by Ectoderm from Another Part of the Body.

The gill region in an embryo with closed medullary folds is shown in outline in Fig. 1 (*BR*). If this ectoderm is removed and replaced by ectoderm from the flank (*FL*) or any other part except that immediately surrounding the gill region, complete suppression of the operculum and the gills on the operated side (Fig. 2) results. Thus, out of twenty-four cases in which ectoderm from the flank was used, seventeen had the gills entirely lacking. In six cases in which the third gill developed and in one in which the first gill was formed, ectoderm from the host covered the greater part of the gill including the tip.

When the covering ectoderm is taken from the region just behind the gills, *i.e.*, from the pronephric and limb area (*PN*), functional gills develop. This was found in five cases, in three of which all three gills were well developed, while in the two others the first gill was either absent or rudimentary.

When, however, the ectoderm is taken from the ventral mid-region (*CD*), *i.e.*, from the region immediately ventral to the branchial, the result is not so favorable. Out of nine cases, no gills at all developed in four, a functional third gill in one, a functional third and rudimentary second in three, and two functional gills (second and third) in one.

Likewise, when the covering is taken from the anterior part of the head, there is usually a partial development of the gills, involving principally the second and third. The transplanted piece was taken in various ways (Fig. 1, *C*, *C′*, *A*). In general, it may be said that the closer the seat of origin of the graft is to the normal branchial region, the more perfect are the gills that develop. A supernumerary balancer developed in almost all of the cases in which the graft included the normal balancer ectoderm.

When the ectoderm covering the gill region is removed and the wound left uncovered, as it was in the donor embryos in many experiments, the surrounding ectoderm pushes over the wound, covering it usually in twenty-four hours or a little more.

The mortality among these embryos is high, but when they live, gills nearly always develop from this regenerated ectoderm. In some cases the gills formed are normal; in others, there is a reduction in number or in size or both, the first gill being more often affected than the others.

Bilateral Extirpation of Branchial Ectoderm.

As far as the question of the morphology of the gills is concerned, this experiment is in no wise different from the unilateral operations, but physiologically it may prove of greater interest. At present only a few cases are available for study. In three experiments the branchial ectoderm was removed on both sides and replaced by ectoderm from the flank. In two of these cases no gills at all developed, while in one the first gill on the left side, which was partly covered by ectoderm from the host, was rudimentary. In the last-named case the circulation of the blood was never established and the embryo became œdematous. In both of the other cases the circulation was established in the vessels of the yolk, but the heart action became more and more feeble, and after a time the circulation of the blood ceased entirely. Both of these eventually became œdematous also, though not extremely so. One individual, killed fourteen days after the operation, showed marked lack of sensitiveness to tactile stimuli, and responded to a needle prick only with a single jerk. It was apparently unable to swim. The other could execute swimming movements fairly well, though in a somewhat jerky fashion. A fourth case that should be considered in this connection had the branchial ectoderm removed on both sides after which it was allowed to regenerate without replacement from elsewhere. The operculum was formed on both sides but the gills failed to develop except in most rudimentary form—on the right side two rudimentary gills and on the left only a sharp conical process (Fig. 3). Blood circulating in these gills was first observed thirteen days after the operation. The larva was kept under observation for thirty-six days, at which age it was preserved. Its development, apart from the absence of gills, was normal.

The experiments are too few in number to warrant very definite conclusions. Absence of gills from both sides seems, in some cases at least, to lead to weakened heart action and ultimate stoppage of the circulation. On the other hand, the last case shows that it is possible for the embryo to adjust itself to the almost complete loss of its respiratory organs, sufficient aeration of the blood probably taking place through the general surface of the body.

Change of Orientation of the Branchial Ectoderm.

These experiments were for the purpose of determining whether there is a definite polarization of the ectodermal elements, and, further, whether any specific gill pattern is predetermined in the region. The experiments of the preceding sections already indicate that the latter is not the case.

Transplantations were made in four ways, as was done in the case of the experiments with limbs. The ectoderm was removed from the gill region and replaced by similar ectoderm either from the same or from the opposite side of the body and placed either with the dorsal or with the ventral border of the graft corresponding to the dorsal border of the wound. Besides these a few experiments were made in which the graft was rotated only 90°. The results may be stated very simply, for there is a marked tendency toward normal development in all combinations. Forty-eight experiments were made, twenty-eight of which gave definite results. Thirteen yielded normal gill-complexes, and these were distributed amongst all of the six different orientations tried. In five cases the second and third gills were normal but the first was small; in two cases the first gill was rudimentary while the others were normal; and in three cases the first gill was altogether lacking, the other two being normal. There were only three cases in which the gill-complex was irregular.

From these experiments the conclusion may be drawn that the branchial ectoderm is equipotential, and that there is no polarization of the elements affecting the potencies of differentiation. In this respect the branchial ectoderm of *Amblystoma* is markedly different from that of the anurans studied by Ekman.

In the latter changes in orientation of this layer are followed by corresponding changes in the position of the gills. This lack of axial differentiation in the gill ectoderm of the *Amblystoma* embryo lasts, however, for a brief period only, for in slightly later stages (from stage 29 on) change in orientation of the ectoderm is followed by marked disturbances in the arrangement of the gills.

REMOVAL OF THE MESODERM OF THE BRANCHIAL REGION.

No satisfactory method of removing the mesoderm of the gill region and replacing it by other mesoderm has been worked out. However, it is a simple matter to remove all of this layer from the region in question and to cover the wound with clean ectoderm from the branchial region of another embryo. This affords the surrounding mesoderm an opportunity to wander in and fill the place taken by the original branchial mesoderm. The wound after such operations heals, though not nearly so readily as when there is mesoderm underlying the outer layer.

Sixteen such experiments were made, of which ten were successful. In only two of these cases was there any radical disturbance of the normal development of the gills. The other eight individuals developed approximately normally, though in five of them the heart was affected and the circulation was never established in the embryo. In three cases, however, the circulation was established on both sides, the gills of the operated side remaining smaller than the normal ones. In one the first gill was suppressed.

From the fact that in such a large proportion of cases the development of the circulation was radically interfered with, it seems likely that the removal of the mesoderm must have disturbed the material that goes to make up the heart. Aside from this the effect of the operation is slight. The mesoderm of the surrounding region obviously has the potency to fill in the gap made by the removal of tissue and then to take part in the development of normally formed, though at first smaller, gills.

CHANGE OF ORIENTATION OF THE BRANCHIAL ECTODERM AND MESODERM.

These experiments are similar to those in which the ectoderm alone was turned, differing only in the circumstance that both ectoderm and mesoderm were lifted and implanted together. There is no difficulty in separating the mesoderm from the endoderm, though the healing of the wounds after transplantation is much less regular than when the ectoderm alone is taken. Inasmuch as turning the ectoderm alone has no effect upon the development of the gills, the effect of the transplantation of the two layers together must be due to the mesoderm alone. The respective results are strikingly different after the two operations.

There were sixty-three experiments in which both mesoderm and ectoderm were transplanted. Thirty-seven were positive. These cases were distributed unevenly amongst the different orientations, but the results throughout, except when the graft was from the same side of the body and normally oriented, were extremely variable and difficult to place in distinct categories.

The normally oriented grafts (homopleural dorsodorsal) gave five normal or nearly normal cases and one distinctly abnormal individual in which two gills were irregular and fused together.

In none of the other orientations were there any absolutely normal cases. However, in the inverted grafts from the opposite side of the body (heteropleural dorsoventral) there were three approximately normal individuals out of eleven positive experiments. Of the remaining eight cases, no two have been given the same designation except two which have been classified as very irregular and two others put down as rudimentary. Three gills were distinguishable in five of the eight cases, and only one gill was present in one. In one of the eight cases, and possibly in others, the abnormalities consisted in defects which might have been due to general conditions rather than to any particular disturbance of orientation.

Inverted grafts from the same side of the body (homopleural dorsoventral) gave not a single case, in a total of thirteen, that even approached the normal condition. Two gills were developed in five cases, but they were abnormal in some respects (Fig. 4); in the others only one gill was formed. Two individuals had quite irregular gills and three had rudimentary ones.

In the non-inverted grafts from the opposite side of the body (heteropleural dorsodorsal), of which there were only six available cases, the results were in no two cases exactly alike. In one a nearly normal complex developed. In two others three gills developed, but with abnormalities. The others were still less perfect.

The above results, standing alone, do not lead to any definite conclusion regarding the exact rôle of the mesoderm in the formation of the gills or regarding the nature of the disturbance caused by abnormal orientation of this layer. It is not clear that it is due simply to the reversal of a single polarized axis, such as is the case in the mesoderm of the anterior limb, although the fact that there is a certain tendency toward normal development in the inverted grafts from the opposite side of the body might be taken to indicate that the anteroposterior axis of the elements of this layer is more markedly polarized than is the dorsoventral. The experiments described in the next section point somewhat more clearly to such a conclusion.

Effect of Increasing the Amount of Mesoderm in the Branchial Region. Superposed Grafts.

In this group of experiments the branchial ectoderm was removed without materially injuring the underlying mesoderm, and a piece of tissue of the same size and shape including both mesoderm and ectoderm was healed over the wound. In this way the amount of mesoderm was approximately doubled, there having been only a very slight loss of mesoderm cells through the operation. The grafts were oriented in the four different ways as in the previous experiments.

The group with normal orientation (homopleural dorsodorsal) developed gills that were normal or nearly so in seven cases (Fig. 5), and in only one, in which the three gills were closely fused, was there any marked abnormality.

The inverted grafts from the opposite side of the body (heteropleural dorsoventral) formed in all five cases gills which approached the normal condition. In two of these the gills were distinctly smaller than normal and in the other three there was considerable fusion and some irregularity, though all three gills could be distinguished.

On the other hand, the inverted grafts from the same side of the body (homopleural dorsoventral) showed in all five cases a very defective development of the gills, only one small, irregular or abortive gill being formed in each case.

In the non-inverted grafts from the opposite side of the body (heteropleural dorsodorsal) the results were likewise for the most part irregular or defective. Two fused gills developed in two cases, and in the other three only a single small, irregular or abortive gill was formed.

The results of these experiments show that while the doubling of the mesodermal material has in itself little or no morphogenetic effect, the orientation of the superadded material is of considerable consequence in determining whether aberrations in the development of the gills occur or not. Aside from experiments in which the transplanted material is normally oriented, these disturbances are apparently least marked in the case of inverted grafts from the opposite side of the body, and in this respect there is an approach to the condition found in the mesoderm of the fore limb bud. However, the results of this orientation of the grafted gill mesoderm are not normal with sufficient constancy to term the combination "harmonic", as was done in the case of the limb.

REMOVAL OF BOTH ECTODERM AND MESODERM WITHOUT REPLACEMENT OF EITHER.

This experiment is a by-product of the experiments described in the last two sections. The individuals are the donor embryos of those operations.

With such a large deep wound as is necessary in removing the gill mesoderm, a high mortality was to have been expected. Out of one hundred and six operations, only ten cases survived ten days or longer. Five others were preserved at intervals from an age of two to that of eight days.

The regeneration that takes place after this operation is very imperfect. In four cases a single fairly normal but small gill was regenerated, identified as the first gill in two cases, the second in one and the third in one. In three of these there were, in addition, one or two rudimentary gills. In the other cases either rudimentary gills alone were formed, or else no regeneration of gills took place at all. Blood circulation was established on the operated side in only two individuals. The circulation was defective throughout the embryo in four cases, as shown by the œdema that developed.

The imperfect restitution that takes place in these experiments must be ascribed to the severity of the operation rather than to the removal of anything specifically essential, for when either the mesoderm or the ectoderm alone is removed there is often complete restoration of function and structure.

Transplantation of Branchial Ectoderm.

The branchial ectoderm was transplanted either to the flank or to the anterior part of the head in a few cases, in order to test the potency of this tissue in strange surroundings. In all four individuals of the first group the results were essentially the same. Small nodules, which might possibly be interpreted as incipient gill sprouts, developed, but after a few days they became obliterated. In one case of the second group, where the gill ectoderm was transplanted to cover the eye and mandibular region, no supernumerary gills were formed and even the normal first gill failed to develop. On the other hand in two cases in which the graft was implanted not quite so far forward (Fig. 1, *A*) there were traces of rudimentary gills in the region of the hyoid and mandibular arches respectively. One of these (Fig. 6) showed two supernumerary filaments quite distinctly, in which, however, the circulation failed to become established. In two other cases, operated in a similar way, no supernumerary filaments were observed.

The potency of the gill ectoderm to form gills in any of these abnormal positions is obviously far less marked in *Amblystoma* than in the anuran species studied by Ekman, especially in *Rana fusca*.

Transplantation of Branchial Ectoderm and Mesoderm.

The results of this experiment are scarcely more definite than when the branchial ectoderm alone is transplanted. There is an initiation of development but it soon becomes arrested.

In five cases the whole of the branchial mound, including both ectoderm and mesoderm but no endoderm, was placed directly behind the normal gills, *i.e.*, in the region of or just ventral to the pronephros, the ectoderm and mesoderm having been previously removed. About the fourth or fifth day a marked prominence was present in the region of the graft, and, in two of the cases at least, distinct gill sprouts showed. There was, however, no further development; the grafted tissue gradually flattened out and became reduced to a slight hump or nodule.

In three other cases the graft was placed in front of the normal gills, in place of the material normally constituting the mandibular and hyoid arches. The results were not very different, except that the original gills of the embryo were to some extent disturbed in their development. No gills ever developed in front of the normal ones and in all three cases the balancer was suppressed.

Effect of Lack of Function on the Development of Gills.

It often occurs in experiments with amphibian embryos that the circulatory system does not function properly. Sometimes no movement of the blood can be observed even though the heart does beat. The effect of this upon the gills is noticeable; they never expand fully, and, while preserving their essential character, they have an atrophic appearance.

In experiments on the gills in particular it not infrequently happens that, although the embryo is otherwise normal, the circulation in the operated gills is either delayed or fails altogether to become established. The same atrophic appearance is found in such cases.

When the heart is removed a similar condition ensues as regards the gills, but the effects on the embryo as a whole are much more pronounced. It becomes badly swollen and ultimately dies. Operations of this kind were done upon embryos in stages 33 and 34, *i.e.*, just before the heart begins to beat. It is difficult to remove the heart rudiment completely. There is almost always some regeneration, and pulsation is established, which, however, is usually ineffective and in such cases no blood can be seen in movement in the yolk vessels or through the gills. Nevertheless, the latter grow out, and are normally constituted except for their atrophic appearance. The operculum and the gill plates are also formed. As regards the development of their specific form, the gills, like many other structures, are thus independent of function, though the marks of atrophy in the functionless organs are unmistakable.

Conclusion and Summary.

So far as the present experimental analysis has led, the respective rôles of ectoderm and mesoderm in the formation of the gills are as follows:

The branchial ectoderm is specifically gill forming, but surrounding the gill region, and, more particularly, posterior to it, the ectoderm has the potency to form gills in a diminishing intensity as the distance from the gills increases. This is shown both by transplantation experiments and by regeneration after removal of the branchial ectoderm.

The gill pattern is not, however, laid down in the branchial ectoderm, as is shown by the fact that this same ectoderm, when turned in any way or even when taken from the opposite side, gives rise to normal gills notwithstanding.

The specific pattern must therefore be determined by the deeper layers, but in the absence of experiments upon the endoderm, the exact rôles of mesoderm and endoderm, respectively, cannot be determined. The indications are, however, that the endoderm does not play merely a passive part, as Ekman maintains to be the case in the anurans.

The mesoderm cannot be a well-defined mosaic, for doubling of the material by superposition, provided the orientation of the grafted tissue is normal, does not disturb the normal development of the gills. Abnormal orientation of the mesoderm, however, both after extirpation of the normal mesoderm and in cases of superposition, results in marked aberration from the normal development, showing that this layer is not without axial differentiation. These aberrations from the normal are not specific for any particular orientation, but there is a greater degree of disturbance after the orientations which, in the case of the fore limb, have been found to be disharmonic, *i.e.*, inverted mesoderm from the same side of the head and non-inverted from the opposite side. One of the combinations called harmonic in the case of the fore limb (that in which the graft is normally oriented) gives normal gills, but the other, while yielding a considerable number of cases of approximately normal gills, results on the other hand in a good many irregularities. These circumstances may be taken to indicate that the gill-mesoderm elements are distinctly polarized in an anteroposterior direction and perhaps less markedly so dorsoventrally. The fact that the mesoderm from the periphery of the gill region is capable of forming gills, taken together with the fact that doubling the amount of the material does not disturb normal development, points to the conclusion that this mesoderm is equipotent.

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'18 Experiments on the Development of the Fore Limb of Amblystoma, a Self-Differentiating Equipotential System. Jour. Exp. Zoöl., Vol. 25..

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Explanation of Plate I.

Figure 1. Profile view of an embryo of *Amblystoma punctatum*, stage 21. The various flaps of ectoderm used in the transplantations are indicated by ovals. A, includes the mandibular, hyoid and anterior part of the branchial region; BR, branchial region; C, ventroanterior part of head region; C′, head region; CD, cardiac region; Fl, flank; PN, pronephric and limb region. × 10.

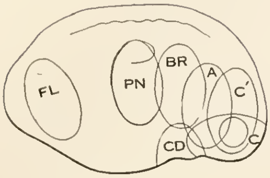
Figure 2. Replacement of branchial ectoderm by ectoderm from the flank (Exp. E. Br. E. 21) resulting in complete suppression of gills on the right side; ventral view of larva eighteen days after operation. × 10.

Figure 3. Larva with gills almost suppressed through failure to regenerate after removal of branchial ectoderm (Exp. E. Br. M 15–); specimen preserved thirty-six days after operation. × 10.

Figure 4. Larva with abnormal gills on the right side due to inversion of the branchial ectoderm and mesoderm (Exp. Br 35), specimen preserved fifteen days after operation. × 10.

Figure 5. Individual showing normal gills developed from double quantity of mesoderm (Exp. S. Br. 17); specimen preserved ten days after operation. × 10.

Figure 6. Individual in which branchial ectoderm (Fig. 1, BR) had been transplanted to a position just anterior to gill region (Fig. 1, A) (Exp. Br. A 2); two supernumerary gills (H and M) developed; the dotted line indicates the boundary of the transplanted ectoderm; sketch made ten days after operation. × 10.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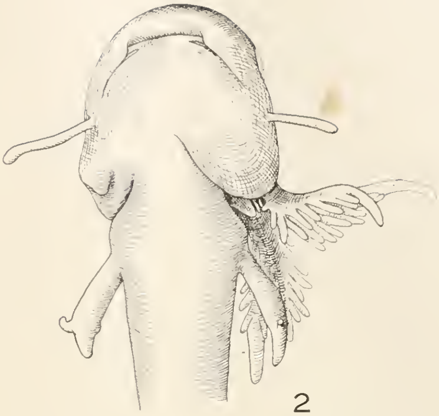

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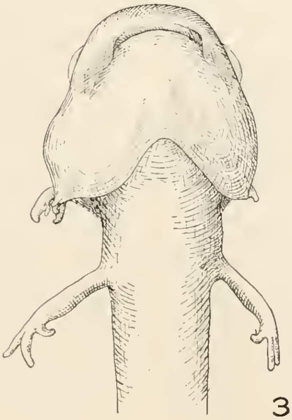

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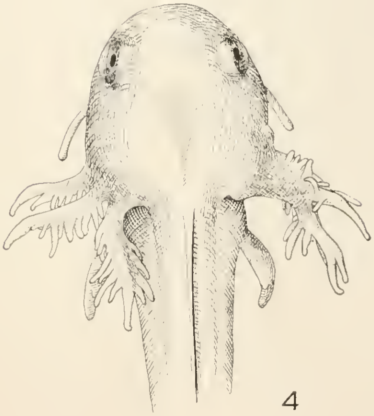

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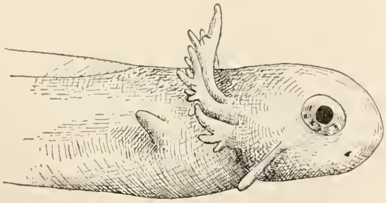

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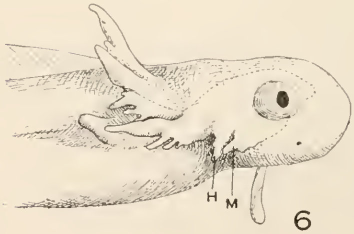

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THE STRUCTURE, LIFE HISTORY, AND INTRAGENERIC RELATIONSHIPS OF PARAMECIUM CALKINSI, SP. NOV.

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Osborn Zoölogical Laboratory, Yale University.

(SEVEN FIGURES.)

The term *Paramecium* was coined by Dr. John Hill of London in 1752 for a group of animalcules comprising "four species," two of which probably include members of the genus *Paramecium* as recognized today.[1] Then O. F. Müller in 1773[2] named an organism *Paramæcium aurelia*, and in 1786,[3] in the first comprehensive attempt to classify scientifically microscopic animals, definitely established the genus *Paramecium* and included under it five species. Unfortunately, however, without the slightest warrant etymological or otherwise, Müller changed the spelling of *Paramecium* to *Paramæcium*. Among the species which Müller distinguished are *Paramæcium aurelia* and *Paramæcium versutum*. The former apparently is Hill's "species 3" and either the present-day *P. aurelia* or *P. caudatum*. *Paramæcium versutum* possibly is Hill's "species 1" and apparently the present species *P. bursaria*. Müller's other paramecia have since been relegated to other genera.

Ehrenberg, in his great monograph on the Infusoria[4] (1838), recognized eight species under the genus and returned to the spelling of Hill with the note "Paramecium ist richtiger als Paramæcium." Among Ehrenberg's species are two true paramecia: *Paramecium aurelia*, which he assumed to be the *P. aurelia* of Müller, and *P. caudatum*, which he distinguished from *P. aurelia* chiefly by the greater attenuation of the posterior end of the animal. Müller's species *P. versutum*, to which Focke in the meantime[5] had given the name *Paramecium bursaria*, Ehrenberg placed under the genus *Loxodes* as *L. bursaria*.

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[1] "History of Animals." London, 1752.

[2] "Vermium terrestrium et fluviatilium Historia." Havniae et Lipsiae, 1773.

[3] "Animalcula Infusoria fluviatilia et marina." Havniae, 1786.

[4] "Die Infusionsthierchen als volkommene Organismen." Leipzig, 1838.

[5] "Ueber einige Organisationsverhältnisse bei polygastrischen Infusorien und Rädertieren." *Isis*, 1836.

In 1841 Dujardin[6] recognized only two species of *Paramecium*, *P. aurelia* and *P. caudatum*, and placed *P. versutum* of Müller (= *P. busaria* of Focke = *Loxodes bursaria* of Ehrenberg) in the genus *Bursaria*, though apparently with some hesitancy.

Claparède and Lachmann in their series of studies (1858–1860)[7] distinguished eight species of *Paramecium*, including *P. putrinum*, a new species which is still recognized, *P. bursaria* and *P. aurelia*. They abolished *P. caudatum* with the remark "Le *P. caudatum* Ehr. n'en est certainement pas spécifiquement différent. C'est une simple variété qu'on pourrait même à bon droit considérer comme la forme typique de l'espèce." [8]

Thenceforth, for about thirty years, the name *Paramecium aurelia* was applied indiscriminately to both *P. aurelia* and *P. caudatum*. Most of the pioneer studies on conjugation, etc., were made, as we know now, on *P. caudatum*, though it was called *P. aurelia*. Then came the first hint which was to straighten out the matter. Maupas in 1883[9] noted a difference in the micronuclear apparatus of certain paramecia which led him in 1888[10] to announce definitely that the structure and number of the micronuclei is a distinctive character and thus on this basis to reëstablish the two common species of *Paramecium*. Since 1889, when both Maupas[11] and Hertwig[12] studied the nuclear phenomena during conjugation of these forms, *P. aurelia* and *P. caudatum* generally have been regarded as distinct species, and the validity of this conclusion has been emphasized by Jennings and Hargitt,[13] and by Woodruff[14] using pedigree culture methods.

Studies on the conjugation phenomena of *P. bursaria* by

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[6] "Histoire naturelle des Zoophytes, Infusoires, etc." Paris, 1841.

[7] "Études sur les Infusoires et les Rhizopodes," Geneva, 1858–1860.

[8] *Ibidem*, part I., p. 265.

[9] "Contributions a l'etude morphologique et anatomique des Infusoires cilies," *Arch. de Zool. Exp. et Gen.*, (2), I., 1883, p. 660.

[10] "Sur la multiplication des Infusoires cilies," *Arch. de Zool. Exp. et Gen.*, (2), 4, 1888, p. 231.

[11] "Le rajeunissement karyogamique chez les cilies," *Arch. de Zool. Exp. et Gen.*, (2), 7, 1889.

[12] "Ueber die Konjugation der Infusorien," *Abh. Kgl. bayr. Akad. d. Wiss.*, München, 2, Cl. 17, 1889.

[13] "Characteristics of the diverse races of *Paramecium*," *Journ. Morph.*, 21, 1910.

[14] "*Paramecium aurelia* and *Paramecium caudatum*," *Journ. Morph.*, 22, 1911.

Hamburger[15] and of *P. putrinum* by Doflein[16] apparently leave no question in regard to the validity of these species, though both species are remarkably similar.

In addition to *P. aurelia*, *P. caudatum*, *P. bursaria* and *P. putrinum*, which are accepted by Schewiakoff in his monographic survey of the holotrichous Infusoria,[17] there must be mentioned *Paramecium trichium* described by Stokes[18] in 1885, and *P. multimicronucleata* by Powers and Mitchell[19] in 1910.

Stokes discovered *P. trichium* in the zoöglœa on an infusion of dead leaves. His description, in part, is as follows:

"It seems to be a distinct species, and one that can scarcely be mistaken for any known form, except possibly for *P. bursaria* (Ehr.) S. K., differing from the latter, however, conspicuously in form, especially in the apparently oblique curvature of the anterior extremity, in the absence of the truncation of the same part, the absence of the rapid and continuous circulation of the endoplasmic contents, and particularly the green coloration of the cortex and sarcode. The two contractile vesicles, instead of being placed one in each body-half, as in *P. bursaria*, are here anterior and close together, contracting quickly, the one beginning to again form almost before the completion of the other's systole. Trichocysts are very abundant, and are so arranged that they seem to elevate the cuticular surface into the minute hemispherical bosses that cover the entire body. When forcibly extruded through the influence of the glycerole of tannin, the distal end of each, for about one tenth of the entire length, is conspicuously thickened, so that the trichocyst seems to be supplemented by the addition of a minute pyramid."

Schewiakoff considers *P. trichium* to be identical with *P. putrinum*, but if Stokes' description is accurate there is no warrant for this conclusion. Certainly further data are needed before a final answer can be given.

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[15] "Die Konjugation von *Paramecium bursaria* Focke," *Arch. f. Protistenk.*, 4, 1904.

[16] "Lehrbuch der Protozoenkunde," 2 Auflage, Jena, 1909.

[17] "Organization et classification des infusoires *Aspirotricha* (*Holotricha auctorum*)," *Mem. Acad. Imp. Sci.*, St. Pétersbourg, Classe d. Sci. Phys. et Math., (8), 4, 1896.

[18] "Some New Infusoria," *Amer. Naturalist*, XIX., 1885.

[19] "A New Species of *Paramecium* (*P. multimicronucleata*) Experimentally Determined," BIOL. BULL., 19, 1910.

P. multimicronucleata, as the name indicates, characteristically has many small micronuclei, usually from six to nine, the structure of which clearly resembles that of the micronuclei of *P. aurelia*. The authors' figures show a general cell-form which is more or less intermediate between that which is typical for *P. aurelia* and for *P. caudatum*. Powers and Mitchell emphasize the persistence of the specific characters after conjugation. It is an interesting form which warrants further study, especially in view of the fact that we now know that reduplication of micronuclei occurs during endomixis.

In January, 1919, I isolated a ciliate, from an infusion of fresh water and vegetable débris collected at New Haven, which, though undoubtedly a *Paramecium*, showed characters that distinguished it from any of the species hitherto described. It has now been extensively studied in pedigree cultures for more than a year and it has bred true. I have, therefore, definitely designated it a new species, and named it *Paramecium calkinsi*[20] in recognition of the fact that Professor G. N. Calkins of Columbia University introduced students of the Infusoria to exact daily isolation pedigree culture methods. *Paramecium calkinsi* presents an interesting composite picture of characters of *P. aurelia*, *P. bursaria*, *P. putrinum* and *P. trichium*, with certain distinctive ones of its own.

The general body form of *Paramecium calkinsi* is more similar to that of *P. bursaria*, *P. putrinum* and *P. trichium* than to that of *P. aurelia* and *P. caudatum*. Indeed, at first glance, one is reminded of *P. bursaria*, and further study suggests *P. putrinum* and *P. trichium* because individuals in different stages of nutrition and dividing at various rates exhibit quite a different appearance. The form which is most characteristic—in fact diagnostic—when the living cells are studied day after day in pedigree cultures is given somewhat diagrammatically in Figs. 1 to 4.[21]

The cells of this pedigree culture of *P. calkinsi*, under favorable food conditions, average about 120 μ in length and 50 μ in breadth. In other words, the length is about the same as that of many races of *P. aurelia*, but the breadth is proportionally greater and the broadest part is anterior, whereas it is posterior to the middle in *P. aurelia*. But, of course, no emphasis can be placed on size relations since it is probable that when *P. calkinsi* from other localities is studied, racial size differences will be found to occur.

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[20] L. L. Woodruff, "*Paramecium calkinsi*, sp. n.," *Proc. Soc. Exp. Biol. & Med.*, Feb., 1921.

[21] Drawings made by Miss J. E. Lovett of this Laboratory.

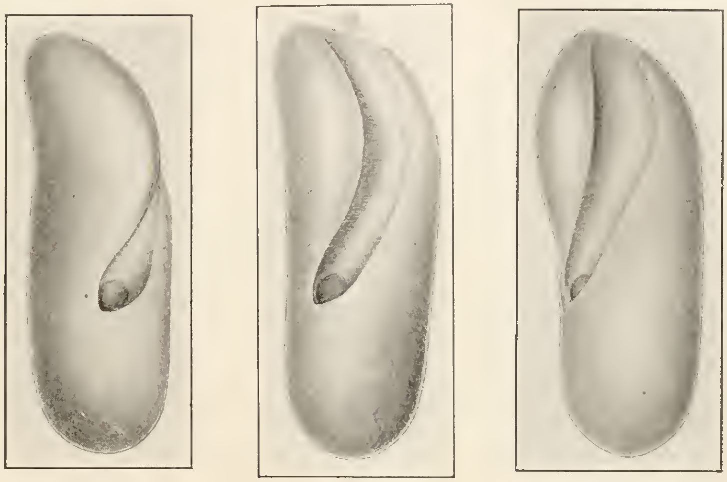

FIG. 1. FIG. 2. FIG. 3.

FIGS. 1–3. *Paramecium calkinsi*, sp. nov.

The ciliary apparatus of the new species affords no striking characteristic and an undulating membrane is present in the gullet as in all paramecia. The trichocyst apparatus is particularly well developed. The trichocysts are relatively evenly distributed in the cortical layer of protoplasm, except at the anterior end where they are more closely crowded together. The surface of the cell in profile presents, upon careful study, a slightly crenulated outline, each of the tiny elevations representing the position of a trichocyst and the depressions between affording the points of origin of the cilia. The structure of the trichocysts as they appear in situ is shown in Fig. 4. Exploded and detached examples appear in Fig. 5.

It will be noted that the trichocyst apparatus of *P. trichium*, as decribed by Stokes, and which he emphasized in naming the organism, agrees closely with that in the new species. The one essential difference is the form of the exploded trichocysts, which in *P. calkinsi* have almost the identical structure of those in *P. caudatum* as described by Khainsky.[22]

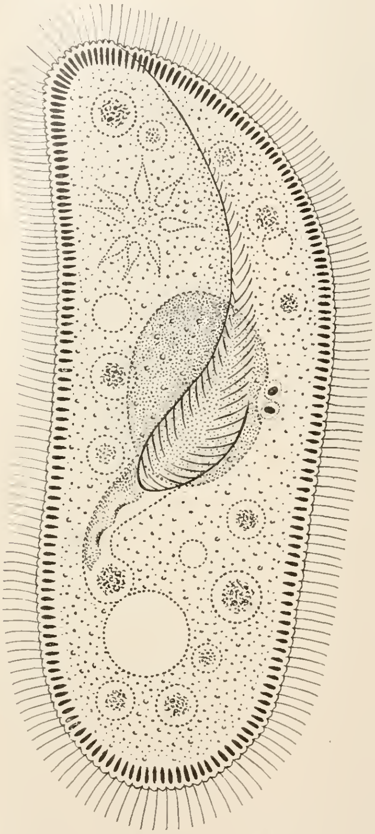

FIG. 4. *Paramecium calkinsi*, sp. nov.

The endoplasm of the new species is characteristically more vacuolated than that of *P. aurelia* or *P. caudatum* when all are bred in the same culture medium. On the other hand it is by no means so fluid as in *P. bursaria*. It is possible to distinguish almost at a glance the new species from the others, merely by the cytoplasmic appearance, when all are mingled together in the same culture.

The contractile vacuoles, as the figure shows, are two in number, and agree closely in position and form with those typical of *P. aurelia*, *P. caudatum*, and *P. bursaria*. Herein is a striking difference from *P. putrinum* with one contractile vacuole at the anterior end and *P. trichium* in which two contractile vacuoles are situated close together near the anterior end.

The macronucleus presents no diagnostic characteristics, though, as the figure shows, in the culture under consideration it usually is proportionately larger in size than it is in the well-known species of *Paramecium*. Little emphasis, however, can be placed on this as the size relations vary in all races of all species under different conditions. But I have never seen the macronucleus of *P. calkinsi* as small as Stokes figured it for *P. trichium*.

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[22] A. Khainsky, "Zur Morphologie und Physiologie einiger Infusorien," *Arch. f. Protistenk.*, 21, 1910.

The micronuclear apparatus is almost identical with that of *P. aurelia* and in marked contrast with that of the other species (see Figs. 4 and 6). In the first place, there are characteristically two micronuclei situated in or near a slight depression of the macronucleus. It has not been possible in every case to discover two micronuclei but there can be no doubt that two are typical of the vegetative cells. And, of course, as in all paramecia, the position of the micronuclei varies considerably in different individuals. Sometimes the macronuclear depression is on one side and sometimes on the other side. Frequently there are two tiny depressions each with a micronucleus. Again, the micronuclei may be at a considerable distance from the macronucleus, though not in mitosis.

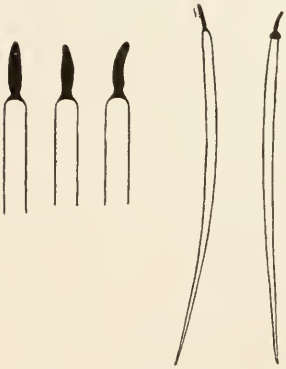

FIG. 5.

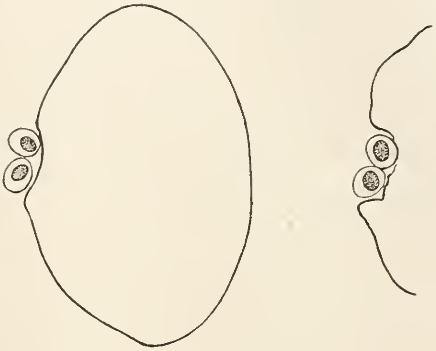

FIG. 6.

FIG. 5. 'Exploded' trichocysts of *Paramecium calkinsi*.

FIG. 6. Micronuclear apparatus of *Paramecium calkinsi*. Left, from a total preparation; right, from a section. Camera lucida sketches.

In the second place, the relative size and the structure of the micronuclei are essentially similar to those of *P. aurelia*. The chromatin of the resting micronuclei is clumped to form an endosome which is surrounded by an 'achromatic' layer enclosed by a distinct nuclear membrane.

The general behavior as well as the 'avoiding reaction,' etc., of *P. calkinsi* is very similar to *P. caudatum* and *P. aurelia*, though the broad and slightly curved anterior end of *P. calkinsi* renders the revolutions on its long axis and its spiral path somewhat more conspicuous than in the more common species of *Paramecium*.

Paramecium calkinsi appears to be adapted to essentially the same environmental conditions as *P. aurelia* and *P. caudatum*. A series of experiments in which flasks of hay infusion were seeded with both *P. calkinsi* and *P. aurelia* showed that, as a rule, one and not both species attained a maximum development in the same flask. Usually within a few days there was a heavy growth of one and only a few struggling survivors, or none at all, of the other species. There was no indication that one was more hardy than the other under the conditions of the experiment; success or failure to dominate the culture apparently depended on which form 'got the start,' so to speak. It seems probable that the excretion products of the two forms are mutually detrimental and that this is the determining factor in the comparative incompatibility of the two species.[23] This point is being investigated.

The rate of reproduction of the new species, as exhibited in pedigree cultures throughout nearly a year, shows no factors which distinguish it from *P. aurelia*, *P. caudatum* or *P. bursaria*, which I have studied under similar conditions, since it is well known that characteristic division rates obtain in different races of the same species. The main pedigree culture of *P. calkinsi* was carried from January 30 to November 20, 1920, when it died out at the 321st generation (Fig. 7). During all this period a constant culture medium of standard beef extract was employed, and during certain experiments the temperature was maintained, by a thermostat, practically constant at 24° C. The race is now thriving in mass cultures which were seeded from the pedigree lines. The animals are apparently in as healthy condition as ever.

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[23] L. L. Woodruff, "The Effect of Excretion Products of Infusoria on the Same and on Different Species, with Special Reference to the Protozoan Sequence in Infusions," *Jour. Exper. Zoöl.*, 14, 1913.

Throughout the year's work every effort has been made to secure conjugation and encystment in the mass cultures, though without result. Furthermore, an intensive study, involving the making of more than a thousand permanent preparations of pedigree animals at all stages in the life of the culture,[24] has failed to reveal any indications of endomixis. Therefore, it can be stated positively that endomixis has not occurred in this culture, although there are fluctuations of the division rate somewhat similar to those associated with endomixis in *P. aurelia* and *P. caudatum*.[25] This point will be considered in detail in another paper.

As is well known, the diet of *Didinium nasutum* is almost exclusively confined to paramecia, and it has not been possible to cultivate successfully the former without this food. An experiment extending through several months showed that *Didinium* thrives on *P. calkinsi* just as well as on *P. aurelia*—further evidence that the new species is a *Paramecium!*

From the foregoing description of the structure and life history of *P. calkinsi* it is apparent that this

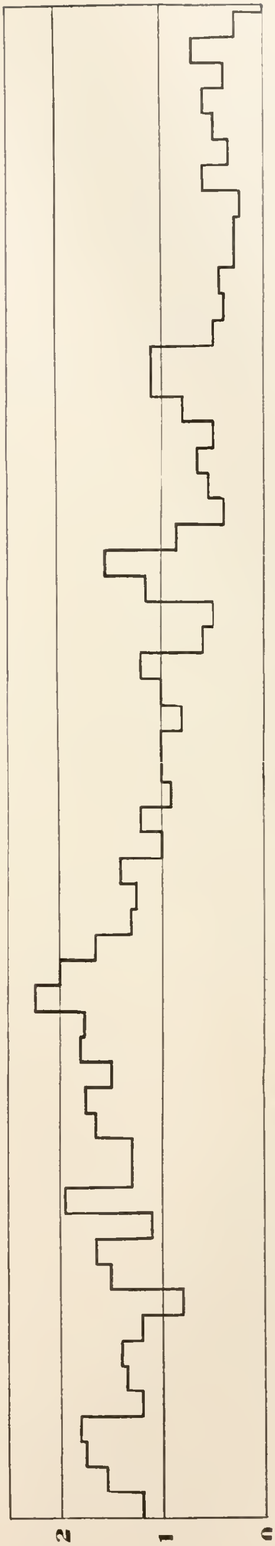

FIG. 7. Graph of the division rate of the main pedigree culture of *Paramecium calkinsi* from January 30 to November 20, 1920. The ordinates represent the average daily rate of division of the four lines of the culture, again averaged for five-day periods.

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[24] I am indebted to Miss Hope Spencer of the Osborn Laboratory for assistance in carrying out this work.

[25] Woodruff and Baitsell, "Rhythms in the Reproductive Activity of Infusoria," *Jour. Exper. Zoöl.*, 11, 1911. Woodruff and Erdmann, "A Normal Periodic Reorganization Process without Cell Fusion in *Paramecium*," *Jour. Exper. Zoöl.*, 17, 1914. Erdmann and Woodruff, "The Periodic Reorganization Process in *Paramecium caudatum*," *Jour. Exper. Zoöl.*, 2[illegible], 1916.

species presents a complex mosaic of the characters of the other members of the genus[26]—indeed the intrageneric relationships of the new species are its chief interest.

A survey of the six species of *Paramecium* under discussion shows that they fall into two quite clearly defined groups which may be referred to as the '*aurelia* group' and the '*bursaria* group' respectively. The members of the '*aurelia* group' (*P. aurelia*, *P. caudatum* and *P. multimicronucleata*) are characterized by a relatively long spindle- or cigar-shaped body; those of the '*bursaria* group' (*P. bursaria*, *P. putrinum*, *P. trichium* and *P. calkinsi*) by a somewhat shorter and broader form, with a tendency, especially prominent in *P. bursaria*, toward a dorsoventral flattening.

Within each of these groups are found two general structural types of micronuclei. One type, which may be termed the '*caudatum* type,' is exhibited by *P. caudatum* in the '*aurelia* group,' and by *P. bursaria*, *P. putrinum* and apparently by *P. trichium* in the '*bursaria* group.' Though Stokes' figure of the micronucleus of *P. trichium* leaves much to be desired, it clearly indicates that the single micronucleus is not of the 'endosome' type. The other type of micronucleus, the 'endosome' or '*aurelia* type,' occurs in *P. aurelia* and *P. multimicronucleata* in the '*aurelia* group,' and in *P. calkinsi* in the '*bursaria* group.' All species with the '*caudatum* type' characteristically possess one micronucleus, and those with the '*aurelia* type,' two micronuclei, except *P. multimicronucleata* in which the number apparently varies from six to nine.

Paramecium calkinsi represents the '*aurelia* type' of micronuclear apparatus in the '*bursaria* group' of species. It is possible that future study may show that certain of the paramecia under discussion (*e.g.*, *P. micronucleata*, *P. trichium*, *P. putrinum*) should be regarded as racial forms rather than distinct species, but I believe that the data presented above indicate beyond doubt that *Paramecium calkinsi* takes its place by *P. bursaria*, as *Paramecium aurelia* has by *P. caudatum*, as a distinct species.

[26] It is unnecessary to illustrate the cell form and micronuclear structure of the several species of *Paramecium* because figures are available in many of the monographs cited. Especial reference should be made to the figures given by Jennings and Hargitt, Schewiakoff, and Hamburger.

March 4, 1921.

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BIOLOGICAL BULLETIN

ATTEMPTS TO CULTIVATE THE BACTEROIDS OF THE BLATTIDAE.[1]

MARSHALL HERTIG.

Since the discovery by Blochmann in 1887 of the bacteroids in the fat-body and eggs of cockroaches, these organisms have been studied by a number of investigators. The bacteroids are organisms very closely resembling bacteria in their morphology and staining reactions. They are found in the fat-body of roaches, filling the cytoplasm of certain cells, the bacteriocytes. They are also found forming a layer over the surface of ovarian eggs and in the yolk of the developing egg. They migrate from the yolk into the fat-cells of the half-grown embryo, where they are found in older embryos, nymphs and adults. The bacteroids have been found in all individuals of nine species of Blattidæ thus far studied, namely, *Blatella germanica*, *Blatta orientalis*, *Ectobia livida*, *Ectobia lapponica*, *Blaberus* sp., *Periplaneta americana*, *Periplaneta australasiæ*, *Parcoblatta virginica* and *Parcoblatta pennsylvanica*. No blattid is known to lack the bacteroids.

The morphology and behavior of the bacteroids in the host tissues are almost identical in all the species studied. The relation of the bacteroids to the host is not understood. The relationship may be merely one of harmless parasitism, there being maintained a delicately adjusted equilibrium between host and parasite, or it may be one of symbiosis, in which both bacteroids and their carriers derive some benefit from the association.

The striking morphological resemblance of the bacteroids to ordinary bacteria and the fact that they multiply within the body of the cockroach have led a number of workers to attempt their culture in artificial media. Blochmann, their discoverer, was the first to attempt their culture in 1887. He employed a number of common laboratory media, as well as a special cockroach-infusion medium, but obtained only negative results. A few years later, Krassilschtschik ('89), according to Cholodkowsky ('91), also attempted the artificial culture of the blattid bacteroids in connection with his work on certain bacteria ("biophytes") found in the bodies of aphids. Though successful in the cultivation of the aphid bacteria, Krassilschtschik failed in his efforts to cultivate the blattid bacteroids. Forbes ('92), in his work on the bacteria normal to the digestive tract of the Hemiptera, stated that he had been unable to cultivate the bacteria found in the fat-bodies of cockroaches. However, Mercier ('06, '07) announced that he had succeeded in obtaining in pure culture on routine media, the bacteroids of *Blatta orientalis*. Tubes of bouillon were inoculated with material withdrawn by means of a sterile pipette from the oötheca, one end of which had been sterilized by flaming. In forty such experiments Mercier obtained pure cultures of an aërobic, motile spore-bearer, which he named *Bacillus cuenoti*. This organism, which grew luxuriantly on all the common laboratory media, resembled closely in cultural characteristics *Bacillus subtilis* and *B. mesentericus*, organisms commonly occurring as contaminators in cultures. Mercier satisfied himself that *Bacillus cuenoti* represented the cultural form of the bacteroids and made certain morphological correlations between the bacteroids and *Bacillus cuenoti*. Mercier obtained cultures from only the single stage, the oötheca, of but one species, *Blatta orientalis*. Javelly ('14) was unable to confirm Mercier's results. Cultures made by him according to Mercier's technique from a number of oöthecæ of *Blatta orientalis* and *Blatella germanica* remained sterile. Javelly concluded that *Bacillus cuenoti* was merely contamination, and that the bacteroids had not been cultivated in artificial media. Quite recently Glaser ('20) has reported the artifical culture of the bacteroids of *Periplaneta americana* and *Parcoblatta virginica*. The organisms obtained by him from both species are spirilla growing readily on routine media. These organisms differ in many important respects from *Bacillus cuenoti*.

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[1] Published with the approval of the Director, as Paper No. 246 of the Journal Series of the Minnesota Agricultural Experiment Station.

In the work of the writer on the bacteroids of the Blattidæ, the results of which are in manuscript, a number of attempts have been made to cultivate artificially the bacteroids of *Blatella germanica*, *Blatta orientalis* and *Periplaneta americana*. The culture media employed include bouillon, agar, gelatin, sugar bouillon (under aërobic and anaërobic conditions), rabbit blood agar, peptone and ascitic fluid-bouillon. In obtaining the bacteroids for inoculation the following technique was employed. Oöthecæ were flamed at one end, a sterile capillary pipette thrust through such flamed surface to the opposite end of the oötheca, thus withdrawing material from within the unflamed as well as the flamed end. This material, which was shown by repeated examinations to contain bacteroids, was transferred to tubes of media. Cultures were also inoculated with bacteroids obtained from the fat-body and ovary. Segments of the abdomen were gently pulled apart by means of forceps, the intersegmental membrane being ruptured. By manipulating the forceps in pulling, one of the segments could be lifted away from the viscera, so that when the rupture occurred the segment would project like a shelf above the digestive tract with its adhering masses of fat-body. With a sterile platinum needle, portions of fat-body were "fished" from the under side of this shelf or from the surface of the digestive tract, care being taken not to touch the torn edge of the segment. By this method remarkably little contamination was encountered. Many cultures remained sterile, or yielded but a few scattered colonies on solid media.

From 20 oöthecæ of *Blatella germanica*, 25 cultures were made. 16 of these 25 remained sterile, while in the other nine there appeared a variety of organisms, none of which resembled the organisms obtained by either Mercier or Glaser. Comparable results were obtained in cultures from the fat-body and ovary. A number remained sterile, while in the other cultures appeared a variety of contaminating organisms. Attempts to cultivate the bacteroids of *Blatta orientalis* and *Periplaneta americana* yielded results entirely similar to those obtained with *Blatella germanica*.

In the endeavor to eliminate contamination entirely, there were made, in addition to the above, a number of cultures using individual bacteroids which had been isolated by the pipette method of Barber ('14). Individual bacteroids obtained from *Blatta orientalis* were placed in hanging drops of peptone or ascitic fluid-bouillon. These bacteroids were charted and observed at frequent intervals. Ten hanging drops were made, the total number of bacteroids in these drops being about 100. No growth whatsoever of the bacteroids or of any other organism took place in these hanging drops. Single bacteria from a laboratory culture placed in control hanging drops multiplied readily. The results of the entire series of culture experiments on the three species of Blattidæ indicate that the bacteroids do not grow readily, if at all, on routine culture media.

It is thus seen that the culture studies of the various investigators have yielded conflicting results. The three earlier investigators, Blochmann, Krassilschtschik and Forbes, did not succeed in growing the bacteroids. Mercier obtained from *Blatta orientalis* an organism closely resembling a number of common contaminators. If the bacteroids could be grown on routine media as readily as *Bacillus cuenoti*, it should be possible to obtain this organism in all cultures inoculated with bacteroids from any stage in the life history of the roach, *i.e.*, from the fat-body, egg or embryo. The work of Javelly and the writer, in which many sterile cultures were obtained, and in which *Bacillus cuenoti* was not found, would seem to indicate that Mercier's organism was itself a contaminator. The results of Javelly and the writer further agree in indicating that the bacteroids of *Blatella germanica* as well as those of *Blatta orientalis* do not grow readily, if at all, in routine culture media.

In Glaser's work with *Periplaneta americana* and *Parcoblatta virginica*, he obtained from each species a spirillum, these two spirilla differing from each other only in minor cultural characteristics. They differ markedly, however, in both morphology and cultural characteristics from Mercier's *Bacillus cuenoti*. Glaser inoculated his cultures with bacteroids obtained from the fat-body of adults. The abdomen was washed with alcohol or a mixture of alcohol and corrosive sublimate, and dissected with sterile instruments. The portions of fat-body containing bacteriocytes were transferred directly to media and incubated. In the writer's experiments with *Periplaneta americana*, three tubes of bouillon were inoculated with bacteroids from three oöthecæ, employing the technique described above. These three cultures remained sterile. A portion of the fat-body of a nymph was obtained by pulling apart the segments of the abdomen as described above, and a tube of bouillon inoculated. No growth occurred. In making a second transfer from this same nymph, the digestive tract was injured and contamination resulted. No other cultures were made from this species. While the number of cultures made from *Periplaneta americana* was limited, the results are seen to be in perfect agreement with those obtained by both Javelly and the writer for *Blatella* and *Blatta*. If the spirillum obtained by Glaser from *Periplaneta americana*, and which grows so readily in a great variety of routine media, actually represents the cultural form of the bacteroids of this species, it is difficult to account for the consistently sterile cultures obtained by the writer.

It was not stated by Glaser whether his technique yielded pure cultures of the spirilla in all cases, or whether contaminations were encountered. The subject of contamination was not discussed, either as concerns organisms in the cultures other than the spirilla or as to the possibility of the spirilla themselves being organisms other than the bacteroids. In view of the conflicting results of various investigators and the small size and filthy habits of the cockroach, the matter of possible contamination would seem to be a paramount consideration. In Mercier's work the flaming of the oötheca did not prevent contamination, and indeed, from his control experiments in which he obtained sterile cultures from the clear liquid bathing the eggs, it would appear that *Bacillus cuenoti* was obtained from within the egg. Javelly obtained contamination in cultures from the fat-body. In the writer's work, contamination was encountered in cultures from both oöthecæ and fat-body. It would seem impossible entirely to avoid contamination, except when employing single bacteroids isolated by the Barber or equivalent technique. The possibility of organisms other than the bacteroids occurring within the tissues or body cavity of the roaches is not precluded, since all the writer's specimens of *Periplaneta americana* were heavily infected with a gregarine, *Diplocystis* sp., inhabiting the body cavity. Glaser did not mention any control experiments showing that the washing of the roach with alcohol or other reagents was successful in sterilizing the surface of the roach. It is conceivable that such reagent might fail to sterilize the areas where one segment overlaps another. In referring to the definite wanderings of the bacteroids in the body of the roaches and in the yolk of the egg, Glaser stated that the organisms in the species studied by him were motile. It is not clear whether motility of the bacteroids was observed directly, or whether the motility of the spirilla in cultures was referred to. In the writer's work many fresh preparations of the bacteroids obtained from five species of Blattidæ, including *Periplaneta americana*, were studied. No suggestion of motility was observed in any case. In view of the conflicting evidence and the technical difficulties involved, Glaser's evidence that the spirilla represent the bacteroids in culture would seem to be inadequate.

As concerns *Blatella germanica* and *Blatta orientalis*, the work of Javelly and the writer indicate quite definitely that the bacteroids do not grow on routine culture media, and that *Bacillus cuenoti* of Mercier is a contaminator. The entirely negative results of the writer in the culture of the bacteroids of *Periplaneta americana*, combined with the evidence from the other species, would seem to furnish strong presumptive evidence that the spirillum obtained by Glaser does not represent the cultural form of the bacteroids of this species.

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BIFURCATION IN THE EMBRYOS OF TUBIFEX.[1]

PAUL S. WELCH.

Introduction.

In the course of some experiments upon one of the common tubificids, *Tubifex tubifex* (Müll.), teratological phenomena were discovered in the early development which merit attention. Instances of bifurcation have been reported in collections of mature and semi-mature Oligochæta from natural environments, but always as great rarities and usually assumed to be the result of regeneration following some form of injury. A considerable number of bifid forms have also been produced in connection with regeneration experiments. Bifurcation as an embryonic phenomenon in Oligochæta has heretofore been known only from six records. According to Kleinenberg (1879, p. 219) and Vejdovsky (1888–1892, p. 250), Dugès (1828) observed and figured a double monster of "*Lumbricus trapezoides*," and Ratzel and Warschawsky (1868) described a similar abnormality in "*L. agricola*." Kleinenberg (1878; 1879, pp. 216–219) found bifurcating embryos not infrequent in the cocoons of "*Lumbricus trapezoides*." Vejdovsky (1888–1892, p. 252) found two such monsters in the cocoons of "*Lumbricus terrestris*," one instance in "*Allolobophora fœtida*," and a large number in "*Allolobophora trapezoides*." Korschelt (1904, pp. 257–258, Pl. 13, fig. 1) described and figured a bifid embryo taken from a cocoon of "*Allolobophora subrubicunda*." Weber (1917) studied the anatomy of twelve double embryos of "*Helodrilus caliginosus trapezoides*" although apparently others were found as indicated by the statement (p. 347) "Out of the 184 cocoons opened 57 contained only one individual, 101 two, and 25 eggs in various cleavage stages and 1 four embryos. Thirty-five of the 101 cases were in the form of monsters."

In order that the identity of the above-mentioned annelids be understood it is necessary to consider the present status of some of the old names just quoted. In connection with the following synonymy it should be noted that at present authorities on Lumbricidæ do not entirely agree as to whether *Helodrilus* or *Allolobophora* should stand as the name of that genus.

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[1] Contribution from the Zoölogical Laboratory, University of Michigan.

Helodrilus fœtidus (Savigny).
    Allolobophora fœtida (Savigny).
Helodrilus caliginosus trapezoides (Dugès).
    Lumbricus trapezoides Dugès.
    Allolobophora trapezoides (Dugès).
Helodrilus subrubicundus (Eisen).
    Allolobophora subrubicunda Eisen.
Lumbricus terrestris L., Müller.
    Lumbricus agricola Hoffm.

It thus appears that several of these writers worked on the same species under different names. Failure to recognize this fact has evidently led Weber into the error (p. 339) of listing "*Lumbricus trapezoides*" and "*Allolobophora trapezoides*" as separate species. The same writer (p. 347), in discussing the difference of opinion expressed by Kleinenberg and Vejdovsky as to the origin of these monsters, suggests that such a difference "may be explained on the basis of the difference in the forms worked on by these two investigators," evidently regarding the species as different, although Vejdovsky (p. 11) states that the material was the same: "Aus meinen embryologischen Untersuchungen ergibt sich ferner, dass die genannte Art mit dem 'Lumbricus trapezoides,' deren Entwicklung *Kleinenberg* bearbeitete, identisch ist."

Only three of the above-mentioned reports were founded upon an appreciable number of bifid embryos, these three pertaining to the same species. The other records were based upon distinct rarities. All recorded instances fall within the Lumbricidæ, the highest family of the Oligochæta. The present paper presents the first account of embryonic bifurcation in one of the lower families (Tubificidæ) of the Oligochæta, these anomalies exhibiting remarkable frequency of occurrence and diversity of form.

MATERIAL.

The material on which this paper is based was identified by Professor Frank Smith as *Tubifex tubifex* (Müll.). Certain localities near Ann Arbor, Michigan, furnish an abundant supply of this annelid, and it has been possible, during the past three years, to obtain large quantities of sexually mature worms and cocoons from November until July either in the native environment or in cultures in the laboratory. Easy access to such an extensive supply of material has made possible the large number of examinations referred to in this paper.

Owing to the abundance of material it was often possible to secure cocoons easily by merely bringing masses of the mud into the laboratory, spreading it out in large shallow dishes, covering it with a small amount of water, allowing the finely divided matter to settle, and gently working over the material with a pipette. Fortunately the mud does not adhere to these cocoons and they will usually appear clean and distinct when uncovered. Furthermore, their color, which is usually of a very light orange, is such as to offer distinct contrast to the mud in which they occur and except for the rough resemblance to the small sand grains sometimes mingled with the mud, the recognition of the cocoons offers no particular difficulty. For securing large numbers of cocoons quickly the writer has employed the method of running quantities of pond and river margin materials through a set of graded sieves, the finest one having a mesh just small enough to retain the cocoons. The residue was then thoroughly washed by a gentle stream of water and examined under good light conditions. Undue handling of materials sometimes results in the loss by the cocoons of their ability to maintain perfectly clean external surfaces, fine particles tending to adhere and thus render cocoons difficult to detect.

Since this work was largely concerned with certain abnormalities in development the cocoons were handled as carefully as possible. That the sieve method of collection was not responsible for the production of abnormalities was demonstrated by comparing the materials so collected with those taken simultaneously from the same habitat by other methods.

Ordinarily, cocoons were carried through their development in the laboratory by merely transferring them to shallow dishes containing river or pond water. For convenience of examination such water was sometimes filtered, although this procedure is not always necessary since the finely divided matter will usually settle and has the advantage of serving as food material for the young worms after they emerge. Occasionally mould attacked the cocoons but its appearance was not frequent enough to offer serious difficulty.

The Cocoon.

Dixon (1915, pp. 85–86) has described briefly the cocoon of "*Tubifex rivulorum*," a form now regarded as identical with *Tubifex tubifex*. In practically all respects this description has been found correct. However, the color of the cocoons demands comment. Dixon describes them as "Whitish or greyish in colour and semi-transparent, but when viewed with the naked eye they appear opaque, but this is due to the eggs which they contain." In this work it has been found that the external appearance depends somewhat upon the degree of development of the eggs within. When first deposited they may have a whitish or creamy tinge but as development proceeds there is an increasing amount of yellow or orange, the maximum intensity being reached at the time when the young worms are ready to escape. At all stages the cocoon proper (the surrounding capsule) is colorless and transparent.

Excluding occasional malformed cocoons, the shape varies from ovoid to sub-spherical with two symmetrically formed, cylindrical, projecting necks, one at each end of the major axis. These necks bear an important relation to certain matters considered in this paper since they are the exclusive avenues of escape for the young worms. At the time of deposition these necks are completely filled by plugs of material somewhat similar in appearance to the remainder of the cocoon but distinctly less resistant since they are easily penetrated at the proper time, while in the hundreds of cocoons examined not a single one had been punctured or ruptured elsewhere although they were often subjected to considerable pressure by developing worms within. The presence of the plugs indicates that no emergence has yet occurred. These plugs vary in form in different cocoons but are usually cone-shaped at both ends, the outer end extending beyond the distal margin of the neck and the inner end extending into the cavity of the cocoon.

The number of eggs included in a cocoon is subject to considerable variation. In more than five hundred cocoons studied the maximum was found to be seventeen and the minimum one. Cocoons containing only one egg were rare, but numbers above ten were rather common, although the general average was somewhat lower. Dixon (1915, p. 85) found a maximum of only thirteen or fourteen. The size of the cocoon is somewhat dependent upon the number of eggs present.

Development of the Eggs.

Owing to the transparency of the cocoon, development of the eggs can be followed with some ease. Two distinct steps occur in the progress from the newly formed egg to the free living condition of the resulting worm, namely, escape from the egg membrane, and subsequent emergence from the cocoon. Ordinarily, eggs "hatch" in a very few days by breaking the delicate, transparent egg membrane but escape from the cocoon is subject to surprising variation and since this phenomenon is directly concerned with the problems in hand, special attention was given to it. This variation occurs not only among different cocoons but also among eggs of the same cocoon. Emergence ordinarily occurs during the tenth to the twentieth day but not infrequently the sojourn within is much longer. Delayed emergences are usually, if not entirely, due to some inability to pass through the neck of the cocoon and usually results in the death of the worm, although occasionally escape is finally accomplished.

Dixon (1915, p. 86) was unable to find more than nine young developing from one cocoon and thought it probable that in those with an unusually large number of eggs certain eggs only develop, the others ultimately deteriorating. Attention was given to this matter and the occasional failure of certain eggs to develop was confirmed. However, more than nine worms emerging from one cocoon were often observed, the maximum number noted being thirteen. Futhermore, there seems to be no way of predicting where the sterile eggs will occur. Apparently they do appear more frequently in the larger cocoons but are not exclusively confined to them. After the development has progressed for two or three days it is usually possible to distinguish eggs which will develop and those which will not since the latter retain for a time their initial appearance thus offering distinct contrast with the changing color and shape of the former.

These sterile eggs have at least two possible fates: (1) They seem to be consumed as food by the developing worms in the same cocoon whenever one or more have been delayed in their escape. Often every trace of such eggs is eventually obliterated. (2) Sometimes all of the developing worms escape early, leaving sterile eggs behind. Such cocoons soon swarm with microscopic organisms which facilitate the dissolution and disappearance of the contents.

Possibly these sterile eggs have something to do with enabling worms to live for long periods within the cocoon after completing their embryonic development. However, instances were observed where worms continued to live in that confinement long after all the granular egg contents had disappeared.

As mentioned before, the only avenues of escape from the cocoons are the two necks. After the plugs are removed the diameter of these necks is just sufficient to permit the average, normally formed individual to squeeze through. Departures from the normal body form, especially swellings or bifurcations, constitute very effective barriers and since, as will be shown later, abnormalities are common in *Tubifex tubifex*, many an individual never reaches the outside world. At the proper time for emergence, exploring movements, especially with the anterior end, are exhibited and when the aperture is finally discovered, efforts leading to escape are quickly exerted. Complete exit may require several minutes, even the greater part of an hour under some conditions. Exit seems to be more easily and more quickly made when the cocoon is surrounded by mud or debris than when deposited in water on the botton of a glass aquarium, this advantage evidently being due, in part at least, to the anchorage which the emerging portion of the worm secures against the surrounding materials with its setæ.

ABNORMALITIES.

In the course of this work several different types of abnormalities were found, all of which appeared early in development and were almost exclusively confined within cocoons since their form was such as to prevent their escape. The following forms were noted: (1) Whole body swollen and shorter than normal; (2) anterior region swollen, posterior region normal; (3) local swellings along the body; (4) bifurcations; and (5) combinations of bifurcations with 1, 2, and 3. Since this paper is concerned only with the fourth type, the other forms of abnormality will receive no further consideration.

FREQUENCY OF BIFURCATION.

During the past three years a large number of these bifurcated worms have been found. While it is possible to examine a cocoon and detect bifid individuals previous to any emergence, they can be secured easily and in some quantity by allowing cocoons to develop under as favorable laboratory conditions as possible until most or all of the normal individuals have escaped. Since the abnormalities only very rarely escape they are left behind in the cocoons and are thus easily collected at a later time. Records based upon more than five hundred cocoons indicate that about twenty percent yielded bifurcated individuals.

The maximum number of bifid individuals found in a cocoon was three, one or two being more common. Thus far no relations between the number of eggs per cocoon and the number of bifurcations has been detected, the latter being about as frequent in cocoons with few eggs as in those containing the larger numbers.

FORMS OF BIFURCATION.

The diversity of form of these bifurcations is striking and demands some notice here. While an occasional irregularity was difficult to interpret, most individuals could be classified according to the following analysis, all of the types specified being represented. It should be stated that this analysis is based upon observations on the activities and external features of the living anomalies.

A. Either anterior or posterior extremity bifid—
  1. Bifurcation simple—
    a. Branches equal.
    b. Branches unequal.
  2. Bifurcation compound—
    a. Plane of bifurcation—
      (1) Secondary bifurcation in same plane as primary.
      (2) Secondary bifurcation at right angles to primary.
    b. Equality of bifurcation—
      (1) Parts of primary bifurcation equal; secondary equal or unequal.
      (2) Parts of primary bifurcation unequal, secondary equal or unequal.
B. Both anterior and posterior extremities bifid—
  1. Primary bifurcations in same plane.
  2. Primary bifurcations in different planes.
  3. Either or both bifurcations compound.

In the writer's records anterior bifurcation with normal posterior end occurs about twice as frequently as a posterior bifurcation with normal anterior end. Furthermore, individuals with both ends bifid are almost as frequent as anterior ones. One series of one hundred and ten records taken at random shows the following proportions: forty-five anterior, twenty-four posterior, and forty-one combined anterior and posterior bifurcations. Other records yield somewhat similar results.

In most instances the recognition of compound bifurcations involved no difficulty. Occasionally, however, a specimen appeared which seemed at first sight to be some form of trifurcation, but careful examination demonstrated that some at least were really compound bifurcations; others, however, could not be definitely classified by external examination. Compound bifurcations are not uncommon and a considerable number were observed during the progress of this work.

The length of the duplicated parts is a matter of considerable variation and the only general relation discovered is that the longer ones occur on the posterior end. Up to date, the writer has not found an anterior bifurcation to exceed six somites, while posterior ones may include seventeen somites. Instances were observed in which the posterior branches constituted more than one half the total length of the body. Many anterior bifurcations are only one somite in extent, two and three being also common. Posterior bifurcations may be short, however, comprising but one somite. A trunk portion of several somites extent is always present, no instances having been seen of such narrow connection of duplicated parts as described by Weber (1917) for *Helodrilus caliginosus trapezoides.*

EMERGENCE OF BIFURCATE INDIVIDUALS.

Among approximately four thousand young worms just emerged from cocoons the writer found only ten which exhibited bifurcations. These were all alive and active. Three were slightly bifid both anteriorly and posteriorly; six had slight anterior bifurcations and normal posterior extremities; and one had a very slight anterior bifurcation and a very deep bifurcation of the posterior end.

The discovery of these ten free individuals indicates clearly that while emergence is possible it is really a rarity. Since nine had completely emerged before they were detected, their method of escape is unknown. The remaining one, caught in the act, was emerging backward, the normal posterior end going in advance. This form of escape appeared to be effective, although the bifurcated anterior end gave some trouble in traversing the cocoon neck. This observation suggests the possibility that the above-mentioned individuals with normal posterior ends emerged backwards. Added weight is given to this suggestion by the repeated observation that in the escape efforts of normal individuals the posterior ends were often projected through the cocoon neck. It seems that the escape of the specimen with shallow anterior bifurcation and very deep posterior one of necessity must have been with the anterior end in advance since as will be shown later deep bifurcations present difficulties which are practically insuperable.

The difficulty of escape from the cocoon by a bifurcate individual is due to one or both of two features, namely, the increased bulk of the double end thus exceeding, ordinarily, the diameter of the neck passage, and the split nature of the end causing the frequent situation of one branch projecting through the neck and the other turned up inside the wall—a situation which is prohibitive of escape. Escape by working the way out backward has already been mentioned but that likewise appears impossible when the posterior end is deeply bifurcate. Even the most striking inequality of diameter in the arms of a bifurcation observed constitutes a serious obstacle to emergence. Both anterior and posterior extremities execute searching movements and instances have been found in which a worm with a deep posterior bifurcation had one posterior branch projected through one neck of the cocoon and the other through the opposite neck. Every kind of bifurcate individual manifests movements which in the normal animal would result in emergence and there seems to be no doubt whatever that failure to escape is due entirely to the abnormal body structure.

Viability in Cocoons.

Mention has already been made of the remarkable viability of some of these worms in cocoons and it should be stated in this connection that both bifurcate and non-bifurcate anomalies exhibit this phenomenon. The writer has records of sojourns within the cocoon for varying periods with ninety-eight days as a maximum and while in almost every case the individual untimately died without having escaped, a few of them (even in the record for ninety-eight days) finally emerged. Why escape could occur after so long a period is not known since the cocoon undergoes no observable change, but it is conceivable that such a form living for days after all the available food material apparently has been exhausted might, because of the continued state of inanition, become reduced somewhat in body size, this reduction permitting the animal to pass out through the cocoon opening. Such body diminution is said to occur in certain other animals when subjected to a continued absence of food. Failure to emerge by non-bifurcate individuals was apparently due to abnormal proportions, this condition being attained before the individual was ready to leave the cocoon.

Rearing of Bifurcate Individuals.

Attempts were made to determine whether it is possible for bifurcate individuals to live and develop if assisted in escaping from their cocoons. In order to insure that they had reached a degree of development at least comparable with that of their normal fellows which were escaping and successfully assuming the free-living life, cocoons containing bifurcations were isolated and bifurcate individuals allowed to remain in the cocoons until most or all normal individuals had emerged. Then the former were dissected out with as great care as possible and introduced into a culture identical in composition and operation with the one in which the normal, newly emerged worms were thriving. Normal worms were used in this way as controls. Up to date the writer has been unable to rear a single bifurcation, although the controls were easily maintained. The reasons for this failure are not understood at present.

Absence in Natural Conditions.

The almost complete, if not wholly complete, absence of bifurcate individuals of *Tubifex tubifex* in nature is shown by the fact that in an examination of over 1,000 worms representing various degrees of maturity and immaturity and collected both from cultures and from native environments not a bifid form was found. The literature seems to contain only one record (Abel, 1902) of a bifid tubificid found in nature, but since similar results have been produced artificially in post-embryonic stages, there is no certainty that Abel's record has any significance in this connection since it might have resulted from an accidental injury. Evidently the cocoon constitutes a very efficient device which automatically eliminates the vast majority of abnormalities.

How universal this high frequency of bifurcation is in the development of *Tubifex tubifex* in all of the varied conditions under which it lives remains yet to be determined. Furthermore, no statement can be made at this time concerning the relation of bifid embryos to those extremely rare bifid oligochætes found in nature, although the available evidence leads one to suspect that the former do not survive and that the latter are the result of regeneration following injury.

MORPHOLOGY.

Only a preliminary account of the internal structure of these anomalies, based upon serial sections of a few individuals, will be given at this time. All specimens studied thus far show the trunk portion of each worm to be a double region whose construction would be simulated by opposing intimately the cut surfaces of two similar ventral halves of normal embryos of the same degree of development. The branches of the bifurcate portion, however, lack this doubleness, being constructed on the plan of a normal individual. Evidence of the double character of the trunk portion is most readily exhibited in the setæ bundles, the nervous system, the nephridia and the principal blood vessels.

Sections through bifurcate portions of specimens show in each branch four setæ bundles arranged in the normal fashion with the component setæ so set and curved as to conform with those of normal specimens provided the nerve cord be interpreted as corresponding to the normal ventral one. In the trunk portion of the body eight bundles of setæ occur in sets of four, each group being the reverse of the other in arrangement and each being properly related to one of the two ventral nerve cords. Furthermore, the distribution of uncinate and capilliform setæ is as this interpretation would require.

Throughout the trunk portion two nerve cords, 180 degrees apart, extend without change to points where bifurcation appears. If the anterior end be unbranched, a mass of nervous tissue encircles the pharynx; if on the other hand it be branched each branch bears a cerebral mass and esophageal commissures.

If the bifurcation plane is frontal in position each nerve chain passes unchanged into each of the branches. Certain modifications of this arrangement occur if the plane of division be sagittal, chief among which is the swinging to one side or the other of each nerve cord.

Nephridia are sufficiently developed in these forms to be detected and one pair is normally related to each nerve cord in each somite, that is, two pairs per somite in the trunk portion. In the branches there is only one pair per somite.

In the bifurcate portions, the main vessels of the circulatory system occur in the normal position, but at the origin of the branches, the two dorsal vessels approach each other and are then laterally displaced by the union of the alimentary canals, one occupying a position to the right, the other to the left. A ventral vessel occurs just above each nerve cord.

It thus appears that at least some of these double embryos are of the type representing dorsal union, the type which Vejdovsky (1888–1892, p. 257) evidently referred to as "Die Doppelmissbindung, deren Individuen längs der Rückenseiten verwachsen," and which Weber (1917, p. 342) has also discussed. While none of the other types found by these authors in double embryos of certain Lumbricidæ have thus far been detected in *Tubifex tubifex* it is possible that some or all of them will appear when a more extensive study involving a much larger quantity of material is completed. Such a study is in progress and results will be reported in a subsequent paper. No attempt is made at present to account for the origin of these anomalies.

SUMMARY

1. *Tubifex tubifex* (Müll.) deposits from one to seventeen eggs per cocoon, a few of which are often sterile.

2. Emergence of young worms from the cocoon is exclusively through two oppositely located apertures.

3. Various forms of abnormality commonly appear among the developing worms, chief among which are bifurcations of the body. Approximately twenty percent of the cocoons examined contained bifurcate embryos. This is the first report of bifur-

cation as an embryonic phenomenon in any of the lower famiies of the Oligochæta.

4. Diverse forms of bifurcation involving both extremities of the body occur in either simple or compound form.

5. Of more than four thousand recently emerged worms examined only ten bifid individuals were found. Any departure from normal body form involving increase in diameter constitutes an extremely effective barrier to escape from the cocoon and practically all of the numerous abnormalities are automatically eliminated.

6. While bifid worms may live for long periods of time imprisoned within the cocoons, emergence is a rarity.

7. Attempts to rear bifid individuals have thus far been unsuccessful.

8. An examination of more than one thousand worms from natural habitats and representing various stages of maturity yielded no bifid forms.

9. A preliminary morphological study, based principally on the setæ, the nervous system, the nephridia, and the circulatory system, shows the body region of these monsters to be double in composition while the bifurcate portions simulate the normal tubificid structure.

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OXYGEN CONCENTRATION AS A LIMITING FACTOR IN THE RESPIRATORY METABOLISM OF PLANARIA AGILIS.

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Living organisms have been conveniently grouped into obligate aërobes, facultative aërobes, facultative anaërobes and obligate anaërobes. The criterion for this classification is fundamentally the relation of organisms to the *concentration* of free oxygen. Free oxygen by its concentration effects on the metabolism acts as a limiting factor in determining animal and plant distribution. While the rôle of oxygen concentration in the life of lower plants and animal parasites has been known in a general way for a long time, yet the importance of differences in oxygen concentration as a factor determining the differences in distribution of many invertebrate animals, which we would ordinarily consider aërobic, has not received the emphasis which it deserves. The grouping of organisms into the above four groups is merely arbitrary, for from the small amount of data available it is clear that some organisms stand in an intermediate position between the above groups.

Since the rate of respiration is correlated to such conditions as state of nutrition, size and age, regeneration, cell division and irritability, it is logical to suppose that by comparing the effects of different oxygen concentrations in the different states of nutrition, age, regeneration and so on, valuable information might be obtained which would throw further light on the relation of respiration to this group of correlated processes.

In the present paper three questions will be considered in so far as they apply to *Planaria agilis*: (1) What is the quantitative relation between oxygen concentration and the rate of oxygen consumption? (2) What is the effect of the state of nutrition upon the relation of oxygen concentration to rate of oxygen consumption? (3) What is the relation of oxygen concentration to the rate of carbon dioxide production and irritability in *Planaria agilis*, which ordinarily would be considered aërobic?

THE RELATION OF OXYGEN CONCENTRATION TO THE RATE OF OXYGEN CONSUMPTION.[1]

The change in rate of oxygen consumption by *Planaria agilis* while it is gradually consuming a given quantity of oxygen dissolved in a given volume of water in a stoppered bottle is shown in the curve in Fig. 1. The data for the curve were obtained as follows: Twenty-one bottles of equal volume were filled with water containing the same amount of oxygen. Ten animals of equal size and the same history were placed in each bottle. At the end of different time periods as indicated in the curve three bottles were analyzed for oxygen content and the average taken as the value which is plotted on the ordinate. The animals had starved for over ten days previous to the experiment and therefore had practically a constant rate of respiratory metabolism as shown by Allen ('19, II.).

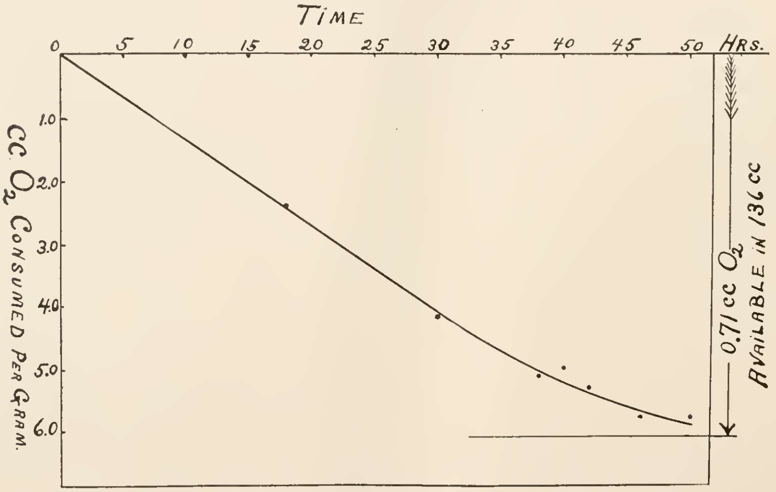

FIG. 1.

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[1] The following is a part of certain studies on respiratory metabolism made possible by a grant from the research fund of the graduate school.

The animals which were used in the experiments reported in this paper were kept at 20° C. during the experiments, and for from two days to two weeks previous to each experiment in order to avoid any acclimation effects due to temperature. The curve shows that the rate of oxygen consumption was constant up to about thirty-eight hours, at which time the oxygen concentration was equal to about one fourth to one sixth of the oxygen concentration of air-saturated water at 20° C. When lower oxygen concentrations were approached the rate decreased. An unused residue of oxygen remained at the end of the experiment. This would have been quickly consumed if the original rate of oxidation had been maintained.

In this method of experiment the possibility that acclimation of the rate of oxygen consumption to lower oxygen concentrations occurs is not excluded. Therefore the following procedure was adopted. If the animals are subjected *suddenly* to definitely known low oxygen concentration and the time period during which they are left in this low oxygen concentration is made brief, then acclimation will probably be avoided. The procedure was as follows. Twenty-eight lots of worms with twenty worms in each lot were selected. All the animals were very closely alike in size, history, etc. The experiment was continued for three successive periods of three hours each. During the first period each one of the lots of animals was placed in a bottle containing air-saturated water. The rate of oxygen consumption per gram worm per hour for this period was determined. At the end of the first period the twenty-eight lots of animals were transferred directly into a second set of twenty-eight bottles containing different but known amounts of oxygen per 136 c.c. water. The animals were now left in these bottles for a second period of three hours. There were fourteen different known concentrations of oxygen. Two bottles were used for each concentration in order to increase the accuracy of the determinations. The fourteen different concentrations of oxygen were as follows: 0.029 c.c., 0.048 c.c., 0.085 c.c., 0.106 c.c., 0.127 c.c., 0.149 c.c., 0.168 c.c., 0.177 c.c., 0.215 c.c., 0.265 c.c., 0.280 c.c., 0.711 c.c., 1.420 c.c. and 1.840 c.c. per 136 c.c. of water.

In this series 0.711 c.c. of oxygen per 136 c.c. water represents the concentration at air saturation at 20° C. The different concentrations of oxygen were obtained by properly mixing known volumes of water of high oxygen content with oxygen-free water.

At the end of the second period of three hours, each lot of worms was again immediately transferred to a bottle containing air-saturated water and left for a third period of three hours. The rate of oxidation per gram per hour during this third period was again determined.[1]

The only results from this experiment with which we are concerned at this time are the rates of oxygen consumption per gram per hour during the *second* period in different concentrations of oxygen. These amounts of oxygen corresponding to the fourteen different concentrations of oxygen are represented by the points on the curve in Fig. 2. Each point is an average of the determinations on two lots of worms. From the curve in Fig. 2 it will be seen that the oxygen concentration which begins definitely

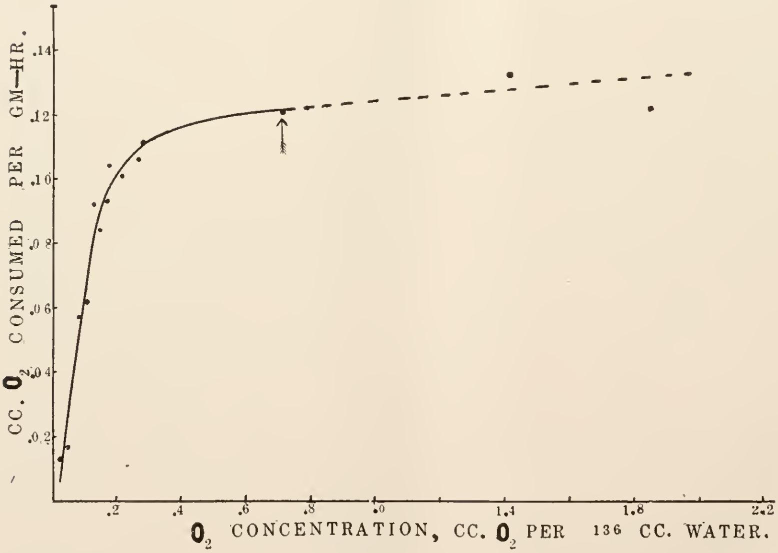

FIG. 2.

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[1] Many precautions and details of technique must be observed in such a relatively complex experiment so that the three-hour period was selected as most suitable. The writer is indebted to Miss Edna Wolf for valuable help in many of the experiments reported in this paper.

to act as a limiting factor on the rate of oxygen consumption lies somewhere around one third and one fourth of the concentration at air saturation, a little higher than that indicated by the curve in Fig. 1. Whether or not the respiratory mechanism of *Planaria* can actually adjust itself by maintaining to some extent a constant rate of oxygen absorption at oxygen concentrations below that which is limiting in such an experiment as the one given above, is an interesting question and will be referred to again.

The question arises as to whether or not *Planaria* can remove the last traces of dissolved oxygen. In general the impression obtained from many experiments of one type or another was, that the very last traces of oxygen could in general not be removed.[3]

The slope of the part of the curve shown by the interrupted line is the part which was left in some doubt in this experiment. Hence several separate tests were made on different lots of animals in order to determine more definitely whether high concentrations of oxygen do increase the rate of oxygen consumption. In each of the experiments two identical lots of animals were tested at air saturation and high oxygen concentration. The following are the results of three separate tests on different lots of worms.

	Concentration of Oxygen at Beginning. C.c. O_2 in 136 c.c. water.		Oxygen Consumed per Gram per 24 Hours.
Exp. I.	0.820		4.02
	1.996		4.38
Exp. II.	0.797	(Air Sat.)	2.92
	1.768		3.36
Exp. III.	1.320		3.88
	2.920		3.74

In general the results from several experiments indicate that there is a slightly higher rate of oxygen consumption at concentrations above air saturation than at air saturation. The approximate amount of this increase is indicated by the slope of the broken part of the curve. The experiment clearly shows that oxygen concentration may act as a limiting factor in the rate of oxygen consumption and shows also what are the quantitative relations.

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[3] Winkler's method as ordinarily used is however not sufficiently accurate as a means of giving an absolute answer to this question, for iodine from potassium iodide is slowly set free by an excess of acid.

This result is similar to those reported by Henze (10) for *Anemonia sulcata*, by Thunberg ('05) for *Limax* and *Lumbricus*, and in the leech as found in this laboratory by Miss Wolf. In a previous paper by Lund ('18) it was shown that the rate of oxygen consumption by *Paramecium caudatum* is practically entirely independent of the concentration of oxygen. This relation would be represented in Fig. 2 by a straight line drawn from some appropriate point on the y-axis parallel to the x-axis. Evidently the oxidation reactions in *Paramecium* and *Planaria* are quite different in certain respects. This is further shown by the fact that the inhibitory action of KNC on the rate of oxygen consumption in *Planaria* is very marked, as shown by Allen ('19, I.), while a similar inhibitory action on the oxygen consumption in *Paramecium caudatum* could not be found, Lund ('18, II.).[4]

It may well be that we shall be able to find in the results of studies on the relation of oxygen concentration to the rate of oxygen consumption, a key to the solution of the question as to why it is that the action of KNC upon *Planaria agilis* and *Paramecium caudatum* is different in character. Work is under way on this question.

It must be kept in mind that since we do not as yet have any accurate data on the magnitude of the diffusion coefficients of oxygen through the protoplasm of cells and different tissues, it is really impossible to say whether in such a case as *Planaria* for example, the decrease in the rate of oxygen consumption at lower oxygen concentrations is due to slow rate of diffusion of the oxygen into the interior of the animal, which rate is itself a function of the difference of oxygen pressure between the inside and outside of the animal, or whether the difference is actually due to an inherent difference in reaction velocity as expressed for example by the velocity constant in the equation for a bimolecular chemical reaction, Krogh ('19). In the absence of appropriate experimental data further discussion of this question is unprofitable.

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[4] Various kinds of criticisms of these experiments have been made by Child and Hyman. A careful consideration of the objections offered has however entirely failed to disturb my confidence in the results as given in the experiments, and the correctness of the conclusions drawn from them. A more complete consideration of the criticisms with further experimental data is given elsewhere. Lund ('21.)

Whatever may be the ultimate explanation or cause of the drop in the curve toward the origin of the axes, such curves for different animals nevertheless may turn out to be useful in interpreting the rôle of oxygen concentration as a factor in determining the distribution of the organism, and if further experiment can show that difference in concentration of oxygen between the outside and inside of the animal does not act as the limiting factor, but rather the concentration of oxygen as such in the cells, then such curves will be very important criteria for the differentiation of different types of respiratory metabolism among animals. A careful comparative study of, for example, the terrestrial, semi-aquatic, and mud-dwelling species of earthworms might throw further light on the question.

The Rate of Oxygen Consumption by Planaria in aërated Water After Living in Water of Low Oxygen Concentration.

Planaria agilis shows a characteristic increase in the rate of oxygen consumption after removal to aërated water from oxygen-free water. This is illustrated in Table I. The experiment was divided into three consecutive test periods. During the first period tests on the seven lots of animals were made in air-saturated water. After eight hours the lots were transferred to a low concentration of oxygen for periods of seven, nine and twelve hours, and at the end of these test periods they were transferred directly into aërated water and the rate of oxygen consumption per gram per hour determined for a third period of four hours. Table I. is a complete statement of the results.

It will be noted that during the third period there is an increase in rate over that in the first period varying from 49 to 85 per cent. There is no proportionality between this percentage increase and the length of time which the animals were subjected to the low oxygen concentration as one might at first expect.

TABLE I.

Planaria starved 26 days before experiment, and kept at 20° C. for 7 days before and during tests. 20 animals in each bottle.

	Period I.—8 hours.			Period II.—7–12 hours.				Period III.—4 hours.		
	Air-saturated Water. C.c. O_2 in 136 c.c. 0.713 0.715 0.715			Water with Low O_2 Content. C.c. O_2 in 136 c.c. 0.033 0.034 0.034				Air-saturated Water. C.c. O_2 in 136 c.c. 0.729 0.736 0.732		
Bottle.	Weight of 20 Worms.	Total O_2 Consumed.	O_2 Consumed per gm. per 24 hours.	Length of Period.	Total O_2 Consumed.	O_2 Consumed per gm. per 24 hours.		Total O_2 Consumed.	O_2 Consumed per gm. per 24 hours.	Per Cent. Increase in Rate of O_2 Consumption.
	gms.	c.c.	c.c.	hrs.	c.c.	c.c.		c.c.	c.c.	
1	.167	.164	2.95	7	.012	.248		.123	4.41	49%
2	.165	.143	2.61	7	.017	.352		.132	4.77	82%
3	.165	.152	2.76	7	.014	.286		.140	5.08	84%
4	.164	.161	2.93	9	.026	.420		.147	5.36	82%
5	.163	.157	2.90	9	.026	.420		.138	5.10	75%
6	.161	.147	2.73	12	.026	.420		.137	5.08	85%
7	.163	.156	2.83	12	.026	.420		.126	4.64	63%

TABLE II.

Planaria starved 14 days before experiment. Temperature 20° C. 11 days before and during tests. Twenty animals in each bottle. Numbers in the table are averages of three bottles. Limit of experimental error 0.010 c.c. oxygen.

		Period I. 6 hours. Air-saturated Water. C.c. O_2 in 136 c.c. **0.720**		Period II. C.c. O_2 in 136 c.c. Bottles 1 to 12, **0.029** Bottles 13 to 21, **0.062**			Period III. 6 hours. Air-saturated Water. C.c. O_2 in 136 c.c. **0.734**		
Bottles.	Total Weight of Worms in Grams.	Total O_2 Consumed.	O_2 Consumed per Gram per 24 Hours.	Length, Hrs.	Total O_2 Consumed.	O_2 Consumed per Gm. Hr.	Total O_2 Consumed.	O_2 Consumed per Gm. Hr.	Increase.
		c.c.	c.c.		c.c.	c.c.	c.c.	c.c.	
1, 2, 3....	0.569	0.42	2.95	1.5	(0.01 −)	0.2 −	0.52	3.67	24%
4, 5, 6....	0.608	0.41	2.71	3	0.02	0.25	0.57	3.79	36%
7, 8, 9....	0.567	0.43	3.04	4	0.04	0.42	0.56	3.99	31%
10, 11, 12	0.593	0.40	2.72	6	0.04 +	0.31	0.55	3.69	35%
13, 14, 15	0.572	0.41	2.85	9	0.13	0.60	0.68	4.75	66%
16, 17, 18	0.560	0.45	3.19	16	0.15	0.41	0.74	5.31	66%
19, 20, 21	0.561	0.46	3.31	24	——	——	0.70	5.00	51%

To test further whether the length of time during which the animals lived in low oxygen concentration had any effect on the percentage increase during the third period, the results of an experiment in Table II. are given. The procedure was the same as that in the experiment, Table I., except that a larger number of bottles were used, with a greater range in the length of the second period, the latter varying from 1.5 hours to 24 hours. Roughly speaking, there is a slightly smaller average acceleration of the rate in the lots of worms subjected to low oxygen for the shorter periods than in the lots left in the low oxygen for the long periods. The difference in the increase is in general quite independent of the length of the period of low oxygen.

Comparison of the Rates of Oxygen Consumption in Air-Saturated Water by Starved and Fed Animals Immediately After Having Been Subjected to Low Concentration of Oxygen.

If we assume that the mechanism which determines the rate of oxygen consumption can undergo an adjustment when subjected to low oxygen concentration, in such a way as to compensate for the inhibitory effect of low oxygen on the rate of consumption, then, would it not be possible to test this assumption by comparing the acceleration, in rate of oxygen consumption, after life in low oxygen, of two lots of animals one of which has been previously subjected to repeated periods in low oxygen concentration, while the other has been manipulated mechanically in exactly the same way without subjection to low oxygen previous to the test?

The data in the columns under lot A and lot B in Table III. give the results of one test. Lot A was subjected to very low oxygen during two alternate periods of eight hours each, while lot B was manipulated similarly except that it was kept in aërated water. The average actual increase in rate of oxygen consumption by twenty animals during the third period is 1.08 c.c. oxygen per gram per 24 hours in lot A, while in lot B it is 1.54 c.c. oxygen per gram per 24 hours. The average increase is therefore 33 per cent. for lot A and 51 per cent. for lot B. A comparison of

Table III.

Planaria starved 7 days before experiment and kept at 20° C. 3 days before and during tests. Each lot of 80 worms was divided into four groups of 20 each for the tests.

		Lot A (80 worms). *Starving.*	Lot B (80 worms). *Starving.*	Lot C (80 worms). *Fed.*
1 Period 8 hrs.......		Low O_2 concentration.	Air-saturated water.	Air-saturated water.
2 Period 12 hrs......		Air-saturated water.	Air-saturated water.	Air-saturated water.
3 Period 8 hrs.......		Low O_2 concentration.	Air-saturated water.	Air-saturated water.
4 Period 44 hrs......		Air-saturated water.	Air-saturated water.	Air-saturated water. Fed liver at beginning of period.
	Bottle.	O_2 Consumed per Gram per 24 Hours.	O_2 Consumed per Gram per 24 Hours.	O_2 Consumed per Gram per 24 Hours.
Period I. 6 hours c.c. O_2 in 136 c.c. = 0.611		c.c.	c.c.	c.c.
	1	3.20	2.75	4.76
	2	3.30	3.09	4.89
	3	3.26	2.87	5.23
	4	3.31	3.36	4.69
	Avg.	3.27	3.01	4.89
Period II. 10 hours c.c. O_2 in 136 c.c. = 0.048	1	.37	.408	.42
	2	.37	.396	.43
	3	.37	.392	.42
	4	.40	.430	.38
	Avg.	.38	.40	.41
Period III. 6 hours c.c. O_2 in 136 c.c. = 0.672	1	4.14	4.61	6.15
	2	4.21	4.50	6.09
	3	4.57	4.37	5.45
	4	4.49	4.73	6.36
	Avg.	4.35	4.55	5.76
Actual average increase...........		1.08	1.54	0.87
Increase............		33%	51%	17%

the same set of twenty animals in each of the bottles 1, 2, 3 and 4 for the first and third periods will show that the increase is similar in all the bottles. For lot A it is 0.94, 0.91, 1.31 and 1.18 for bottles 1, 2, 3 and 4 respectively, while for lot B it is

1.86, 1.41, 1.50 and 1.37 for bottles 1, 2, 3 and 4 respectively. It might seem that the previous life in low oxygen did have an effect which resulted in slightly decreasing the acceleration in aërated water. This therefore, so far as it goes, confirms the inference from the comparison of the curves in Figs. 1 and 2 above in respect to the difference in the limiting concentrations of oxygen in the two methods of experiment used in obtaining the two curves.

A second question upon which the experiment given in Table III. was intended to throw light is: What effect does the state of nutrition have upon the magnitude of the increase of oxygen consumption during the third period? The animals of lot B were treated exactly like those in lot C except that those in lot C were fed liver about forty-two hours before the beginning of the first test period. The expected marked rise in oxygen consumption occurs in lot C during the first period. This rise persists during the third period but it will be noted that the increase in rate during the third period over that in the first period is only 0.87 c.c. per gram per 24 hours in the fed lot C as compared to 1.54 c.c. per gram per 24 hours in the starving lot B. The average increase in lot C is 17 per cent. while in lot B it is 51 per cent. This question was tested further by comparing fed and starved animals. Table IV. is self-explanatory. The animals in bottles numbered 5 to 8 were fed beef liver twenty-four hours previous to the experiment. The results again show a greater increase of oxygen consumption by the starved than by the fed animals, during the third period.

It was suspected that the increased rate of oxygen consumption during the third period might be due to increased motor activity by the animals during the third period. Accordingly in an experiment, the results of which are given in Table V., the heads of both starved and fed animals were cut off after feeding, that is, about 24 hours before the beginning of the experiment. All the data are calculated on the basis of the weights of the animals before feeding after subtracting the weight of the heads which were removed after feeding. The results on such decapitated fed and starved animals show the same relations in respect

Table IV.

All *Planaria* starved 26 days before experiment. Temp. 20° C. 7 days before and during experiment. 18 animals in each bottle.

		Period I. 6 hours.	Period II. 9 hours.	Period III. 6 hours.	
		O_2 Concentration in c.c. per 136 c.c. Water.			
		0.672	0.013	0.674	
	Bottle.	Total c.c. O_2 Consumed During the Period.			
Starved.....	1	0.083	0.005	0.119	
	2	0.100	0.005	0.122	
	3	0.100	0.005	0.116	
	4	0.126	0.010	0.136	
Fed........	5	0.169	0.012	0.183	
	6	0.178	0.015	0.192	
	7	0.185	0.012	0.202	
	8	0.174	0.017	0.173	
		Average, C.c. Oxygen Consumed per Gram Weight per 24 Hours.			
		Period I.	Period II.	Period III.	Increase.
Starved.....		3.030	0.127	3.650	20%
Fed........		5.220	0.204	5.540	6%

to the increased rate of oxygen consumption during the third period, as that found in the whole animals used in Tables III. and IV. The absolute increase during the third period, among the different experiments, is not accounted for by any difference in the length of the starvation periods of the animals previous to the experiments, for these were the same. On the other hand, any differences in motor activity which may have occurred in the different experiments do not seem to me to give any adequate physiological explanation of the effect. The magnitude of the effect of movement on the respiratory exchange may be inferred from certain observations on *Planaria agilis* made by Allen ('19, I.). There remains the alternative explanation, that the increase in oxygen consumption after living in low oxygen concentration is due to the oxidation of lower fatty acids or other by-products

TABLE V.

All *Planaria* starved 33 days before experiment. Temp. 20° C. 30 days before and during the tests. One half of the total number of worms were fed beef liver 24 hours before beginning of the tests. *Heads of all the worms were cut off one hour after feeding.* 20 worms in each bottle.

			Period I.	Period II.	Period III.
			O_2 Concentration in c.c. per 136 c.c. water.		
			0.752	0.030	0.748
	Bottle.	Weight of Headless Worms, gms.	Total c.c. O_2 Consumed During the Period.		
Fed.........	1	0.146	0.207	0.013	0.200
	2	0.143	0.203	0.008	0.215
	3	0.139	0.195	0.010	0.196
	4	0.138	0.212	0.010	0.222
Starved.....	5	0.132	0.106	0.005	0.142
	6	0.133	0.125	0.008	0.116
	7	0.131	0.111	0.002	0.135
	8	0.135	0.115	0.007	0.127

	Average, C.c. Oxygen Consumed per Gram Weight per 24 Hours.			
	Period I.	Period II.	Period III.	Increase.
Fed.................	4.34	0.16	4.44	2%
Starved..............	2.57	0.07	2.93	14%

of catabolism which accumulated during the period of life in low oxygen.[5] In the absence of a complete chemical study of the metabolism of *Planaria* the results await an adequate explanation.

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[5] In his monograph "Respiratory Exchange of Animals and Man," Chapter VI., page 83, Krogh states that, according to Lesser (*Zeitschr. f. Biol.*, vol. 54, pp. 1–17), earthworms after living in the absence of oxygen, when returned to air show an increased rate of oxygen consumption, due to oxidation of accumulated oxidizable residues during the anaërobic period. In fact Lesser found just the opposite and states definitely, pp. 11–12: "Durch Respirationsversuche . . . wurde festgestellt, das der respiratorische Quotient beim Regenwurm . . . in der Erholung nach vorausgangener Anoxybiose erhöht ist gegenüber dem normalen. Es findet mithin in der Erholung eine völlige Verbrennung . . . der früher als Hauptprodukt der Anoxybiose gefundenen Fettsaüre nicht statt."

THE RATE OF CO_2 PRODUCTION IN THE ABSENCE OF FREE OXYGEN.

The rate of carbon dioxide elimination by *Planaria* was determined by the use of a modification of the method described some time ago by Lund ('19). An important improvement in the method was brought about by washing the bottles with a stream of CO_2-free air, after the animals were placed in the bottles, thus doing away with the correction for CO_2 present in the air at the beginning. Where CO_2 production was determined in the absence of oxygen the bottles were washed out with hydrogen. All titrations were performed without removing the stopper from the bottle, by means of the simple expedient of boring two holes, one on each side of the bottle. These holes were fitted with stoppers and titration was carried on through one of these small openings. This procedure simplifies the method and increases its accuracy. The method has been used extensively in this laboratory and has proven itself to be satisfactory, especially when periods of two to three hours or more are used and comparative results are desired.

Table VI. gives the results of an experiment where twelve lots

TABLE VI.

Planaria starved and kept at 20° C. 38 days before experiment. Temperature during experiment 21° C. Twelve bottles with 50 animals in each. Blanks run as controls are not given in the table. 1 c.c. N/100 HCl equivalent to 0.111 c.c. CO_2.

	Duration of Test in Hours.	5	10	15
Air. . .	Total c.c. CO_2 produced per gram during test.	0.645	1.332	2.041
		0.504	1.561	2.241
		Avg. 0.574	1.446	2.141
	Average c.c. CO_2 produced per gram during last 5 hours of test.	0.57	0.87	0.69
H_2. . . .	Total c.c. CO_2 produced per gram during test.	0.708	1.371	2.140
		0.766	1.345	2.077
		Avg. 0.737	1.358	2.108
	Average c.c. CO_2 produced per gram during last 5 hours of test.	0.73	0.62	0.75

of fifty animals in each lot were used. Six bottles were filled with CO_2-free air while another lot of six bottles were filled with hydrogen. The CO_2 produced by the worms in two bottles from each of the series filled with air and hydrogen respectively was determined at the end of 5, 10 and 15 hours, as shown in the table. The amounts of CO_2 produced during each one of the successive periods of five hours in air was 0.57 c.c., 0.87 c.c., 0.69 c.c. CO_2; and in hydrogen 0.73 c.c., 0.62 c.c. and 0.75 c.c. CO_2. The lots of fifty worms each were of course selected carefully as to size, history, etc., so as to render them comparable. The table shows clearly that no marked difference in the rate of CO_2 elimination occurs in the animals in air and those without free oxygen. All animals were normal at the end of the experiment.

Table VII. is a similar experiment of longer duration. The carbon dioxide elimination during the successive four periods of seven hours each is in air 0.80 c.c., 0.95 c.c., 1.47 c.c., 0.95 c.c., while in hydrogen it is 0.64 c.c., 1.06 c.c., 1.27 c.c., 0.21 c.c. A comparison of the condition of the animals at the end of the 7-, 14- and 21-hour periods showed all the animals in hydrogen to have a normal response to light and touch. While at the end of twenty-

Table VII.

Planaria starved and kept at 20° C. 11 days before the experiment. Temperature during the experiment 19.5° C. Twenty-five worms were placed in each bottle in 2 c.c. water. Blanks run as controls are not given in the table. 1 c.c. N/100 HCl equivalent to 0.111 c.c. CO_2.

	Duration of Test in Hours.	7	14	21	28
Air...	Total c.c. CO_2 produced per gram during test.	0.89 0.72	1.65 1.86	2.99 3.45	4.17 4.17
		0.80	1.75	3.22	4.17
	Average c.c. CO_2 produced per gram during the last 7 hours of test..................	0.80	0.95	1.47	0.95
H_2....	Total c.c. CO_2 produced per gram during test.	0.70 0.58	1.84 1.46	—— 2.97	2.95 3.41
		0.64	1.70	2.97	3.18
	Average c.c. CO_2 produced per gram during the last 7 hours of test..................	0.64	1.06	1.27	0.21

eight hours irritability was practically lost in those living in hydrogen. The animals in air were normal. The loss of irritability is clearly correlated to the fall in rate of CO_2 elimination during the last seven-hour period. All animals in this twenty-eight-hour period in hydrogen recovered when removed to air.

Another similar experiment which lasted thirty-eight hours gave similar results. A fall in rate of CO_2 elimination in hydrogen was again associated with loss of irritability and muscular tone. One set of animals in the thirty-eight-hour period only partially recovered when removed to air-saturated water. The controls in air were normal.

It is evident that the loss of irritability and appearance of a condition which closely resembles narcosis is correlated more closely to decrease in rate of CO_2 production than the lack of free oxygen.

An important fact to notice is that the action of KNC and the effect of absence of free oxygen are quite distinct. The experiments above show clearly that the rate of CO_2 elimination is not noticeably changed for hours after free oxygen has been removed. Potassium cyanide in proper concentrations inhibits oxygen consumption to as much as 70 to 80 per cent., as shown by Allen ('19, I.) and confirmed by Hyman ('19), and also markedly inhibits carbon dioxide elimination as found by Child ('19). While the absence of free oxygen does not affect the rate of CO_2 production for many hours, so far as the evidence goes, the return of *Planaria* to air-saturated water from KNC solutions strong enough to cause an inhibition of 50 to 80 per cent. does not result in an increase in the rate of oxygen consumption, after the return to air-saturated water, as is the case after life in the absence of free oxygen. Another fact to be noted is that it has not so far been possible to obtain a complete inhibition of the oxygen consumption in *Planaria agilis* by means of KNC. These differences in the action of KNC and lack of oxygen have not been fully recognized by previous investigators, while it has often been assumed that lack of oxygen and KNC solutions have the same effects.

SUMMARY.

1. Oxygen concentration becomes a limiting factor in the rate of oxygen consumption by *Planaria agilis* at about one third air saturation of water at 20° C.

2. Life in low oxygen concentrations results in an accelerated respiratory metabolism varying from two to eighty-five per cent. when the animals are returned to air. This increase is not proportional to, nor directly dependent upon, the previous length of time which the animals have lived in what practically amounts to an absence of oxygen. Some slight evidence was obtained which indicated that repeated subjection of *Planaria agilis* to low oxygen concentration may result in a small decrease of the acceleration of oxygen consumption when returned to aërated water after living in very low oxygen concentration.

3. The percentage acceleration of oxygen consumption referred to under 2 is more marked in starved than in fed animals.

4. The rate of carbon dioxide elimination continues at practically the same rate in hydrogen as in air, until loss of irritability and muscular tone begins. The loss of irritability in *Planaria agilis* is therefore more closely correlated to change in rate of CO_2 production than to consumption of free oxygen if in fact it is correlated closely to either one.

5. The effect of KNC in inhibiting the oxidations in *Planaria* is not identical with the effects due to the absence of free oxygen.

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THE BEHAVIOR OF CELLS IN TISSUE CULTURES OF FUNDULUS HETEROCLITUS WITH SPECIAL REFERENCE TO THE ECTODERM.[1]

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Introduction.

Human, mammalian, avian and amphibian tissues have been frequently employed for the study of cells in tissue cultures but comparatively few observers (Osowski 1914, Lewis 1916 and Dobrowolsky 1916) have used fish tissue. These embryos are easily obtained, the culture media are simple to prepare, and large growth takes place at room temperature. They therefore constitute an ideal material in which to study the movement of the epithelial membrane, the structure of the ectoderm cells and their relation to other cells.

Technique.

Pieces of *Fundulus* embryos were explanted into diluted sea water in the manner described by M. R. Lewis ('16). As an additional precaution against infection, the eggs were dropped into 95 per cent. alcohol for one second and then transferred to sterile sea water. In preparing the media several dilutions of sea water were employed: *i.e.*, 20 per cent., 30 per cent., 35 per cent., 40 per cent. and 50 per cent. in distilled water. To 80 c.c. of each of these dilutions was added 20 c.c. *Fundulus* bouillon, 0.02 gm. $NaHCO_3$, and 0.5 gm. dextrose. The media were then sterilized.

Growth was obtained in dilutions of sea water ranging from 20 to 50 per cent., although in the latter it was infrequent and not of great extent. While growth was satisfactory in a dilution of 20 per cent., the proportion of good growths was larger with 30 per cent. and still larger with 40 per cent. sea water. Moreover, cell proliferation from each piece was more abundant. Cultures grew in media containing no dextrose, or with varying amounts of dextrose up to 2 per cent. Media containing 0.5 per cent. gave the best results. The cultures grew equally well with 80 per cent. of Locke's solution in place of sea water. Good results were obtained with 80 per cent. of Locke's solution and 20 per cent. of chicken bouillon substituted for the fish bouillon, although not so many cultures grew. Pure egg albumen and dilutions of this added to the normal medium likewise proved successful.

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[1] From the Marine Biological Laboratory, Woods Hole, Mass.

Several cultures which contained tissue from both chick and *Fundulus* embryos were made in Locke's solution, chicken bouillon and dextrose. These were kept in the incubator at 39° C. and at the end of 48 hours the explants showed equally good outgrowths.

Cultures were usually prepared in the afternoon, and by the following morning growths in the form of membranes had formed, though sometimes this did not occur until the second day, especially when a dilution of 20 per cent. sea water was used. The embryos were 7, 10, 14 and 15 days old (just before hatching) but the age made no apparent difference in the growth from the explant.

General Characteristics of the Cultures.

Examination of the cultures shortly after explantation usually showed a few isolated mesenchyme cells, which soon began to migrate outward on the cover-slip. The ectoderm cells in the region of the cut edges rounded up into more or less spherical bodies. After several hours typical cultures revealed a membranous outgrowth of ectoderm from one or more regions of the explant, and beyond this an area of mesenchyme cells, isolated, or forming a reticulum upon the under surface of the cover-glass (Fig. 1). Nerve fibers, projecting freely into the fluid along the coverglass, were often present in abundance. Pigment cells and yolk-filled cells from the digestive tract (Fig. 7) were also common. No outgrowth of muscle cells was observed.

A renewal of the fluid medium was not attempted in any of the cultures. The oldest healthy cultures were ten days old, and in one of these the heart was still beating at the time of fixation. In one culture peristalsis was observed in a portion of the intestine which projected out into the medium. Contractions of the trunk musculature, beating of the heart, and movements of the fins were frequently observed in cultures several days old.

Behavior of Ectoderm Cells at Cut Edges.

In normal embryos the ectoderm consists of a single layer of large pavement cells, polygonal in surface view, which may be clearly seen in the regions covering the fins and the trunk musculature. The cells are transparent and almost colorless. The internal structures are only vaguely visible. The cells covering the trunk have delicate concentric markings, suggesting somewhat the markings upon fish scales.

When the embryos are cut the ectoderm cells along the edge round up very markedly into spherical masses, especially in the region of the heart and the yolk sac. Any small group of cells or single cells that have in cutting become separated from the explant remain for some time in this state. Eventually, large vacuoles develop in them and they remain inactive in this condition for several days before disintegration takes place. Similar cells were frequently observed in old cultures along the edge of the ectodermal membrane.

The direction of the cut is a factor in the successful growth of cultures from the trunk region of the body. The cells do not grow out unless the cut is irregular or oblique. If it is transverse the cells close in and form a covering over the injured end, preventing outward migration. Osowski found that injured surfaces of fish embryos were covered by an epithelial membrane within twenty-four hours.

Membranes never grow out from cut fin surfaces; the cells near the cut surface become wrinkled and irregular in contour, and remain in this condition sometimes for several days, before they become rounded up and display the large vacuoles and greenish protoplasm which are characteristic of degenerating ectoderm cells. Sometimes the ectoderm of the fins degenerates even when not cut, although the other cells of the culture exhibit normal activity.

Ectodermal Membrane.—Fig. 1 shows the extent of a characteristic membrane in a seven-day culture. In the living cultures the ectodermal cells spread out in a very thin, flat and colorless layer on the under side of the cover-glass. The position of the nucleus and of the granules within the cell could rarely be detected. At the edge of the membrane the cells were thicker and darker in appearance, with a greenish tinge and of very irregular form. Projecting beyond the ectoderm, mesenchyme cells could be seen adhering closely to the cover-glass. Other cells, slightly darker and containing vacuoles and granules, were observed migrating above the ectodermal layer. Slides stained with iron hæmatoxylin show that such outgrowths from the explant consist of a practically continuous ectodermal membrane of extremely flat, slightly granular cells, and a more or less imperfect membrane of mesenchyme lying above it, closely adhering to the cover-glass (Fig. 2). The mesenchymal membrane is never so perfect as the ectodermal layer, and there is a gradual transition from a membranous form to more or less isolated cells which may project for a considerable distance beyond the ectoderm (Fig. 3). One group of ectodermal cells was found which was not covered on its upper surface by mesenchyme (Fig. 8). These cells were characterized by very granular nuclei containing one or more nucleoli, and by mitochondria in the form of threads and granules. Frequently the cells showed a rosette-like arrangement around a small intercellular space. Mesenchyme cells were readily distinguishable in the stained slides by their more granular cytoplasm and indefinite cell boundaries. The size of the nuclei was not a criterion, as the nuclei might be either larger or smaller than those of the ectoderm. Mitochondria were much more abundant than in the ectoderm and stained more deeply. Fig. 9 shows a group of mesenchyme cells, unaccompanied by ectoderm, in which the mitochondria appear very clearly. They were also observed in the living cells when stained with janus green. Certain other granules became visible when stained with neutral red. The latter stain also affected granules in the thickened cells at the edge of the ectoderm, but the presence of such granules in the flat ectodermal cells could not be determined with certainty. These cells appeared less easily penetrable to a number of vital dyes, while the mesenchyme cells were readily colored.

Formation of Ectodermal Membrane.—In a study of fetal skin growing in blood serum, Loeb ('12) observed that the ectoderm cells migrated into the surrounding medium in the form of strands. Holmes ('13) and Uhlenhuth ('14) described a similar condition in the frog. In my observations on fundulus the ectodermal outgrowths were invariably in the form of very thin, one-layered membranes. The ectoderm never migrated in strands. The earliest appearance of the membrane was indicated by an exceedingly thin, flat layer near the explant which was continuous with the rounded cells covering the body region, and bordered along its outer edge by a broad mass of irregular thickened cells. The mesenchyme cells lay in a thin sheet above the ectoderm and projected beyond it. As the rounded cells migrated from the explant their contour changed gradually, and they became flattened with irregular, thickened, central portions which projected downward in the fluid medium. Text-figure 1 is a drawing of an ectodermal cell in the process of flattening out during its migration. The portion of the cytoplasm spread out along the cover-glass formed a clear thin area bounded by a cell wall which was in close contact with the walls of neighboring cells. In this clear region were a few pale concentric markings. The central mass of thickened cytoplasm eventually disappeared as the cell flattened out completely. A few hours later, as a result of the migration and flattening of the cells, the membrane had increased more than twice in extent, and no thickened cells remained except a few scattered ones and a single row of elongated cells around the edge.

Changes at the Edge of the Ectodermal Membrane.—The edge of the ectodermal membrane was seen to undergo slow and continual changes during the active growth of the culture. Usually the cells were thickened and elongated or extremely irregular in form, with numerous blunt knobs projecting downward. Their cytoplasm appeared granular in contrast to the apparently homogeneous protoplasm of the flat cells. They were observed to flatten out and thus extend the membrane. It seemed as though the cells were under unequal tension for as the membrane grew wrinkles formed in cells that had formerly been flat. The wrinkles could be differentiated from the cell thickenings previously described, for they were obviously folds in the membrane and often involved more than one cell.

Ectodermal pseudopodia, formed from a hyaline outer region of the cells, have been observed by Harrison ('10) and by Holmes ('13) in tissue cultures of the frog. In the ectoderm of fundulus, however, the entire cell is equally hyaline when stretched out flat, and the formation of pseudopodia was never observed.

The ectodermal membrane is not only very extensible, but elastic as well. In staining the cultures with vital dyes the greatest care was necessary to prevent its retraction, which often followed within a few seconds. This is also likely to occur if the slides are jarred. In one culture the ectoderm cells along the edge contracted and thickened, pulling along with them large portions of the mesenchyme membrane. This double membrane then rolled in upon itself and, as it tore loose from the cover-glass, could be seen adhering by short projections from certain of the cells. These in turn loosened and the membrane pulled in farther (Fig. 5). In such cases of mechanical disturbance of the culture the membrane later became reduced by the contraction of the ectoderm to a compact mass of cells which eventually disintegrated. Ruth ('11) describes the contraction of the edges of the growing epithelial cells during the healing of a wound in the skin of a frog in vitro.

Striations in Ectoderm Cells.—The most peculiar characteristic of the ectoderm cells is the presence of numerous delicate striations, more or less concentrically arranged, which form an intricate pattern over all the cell (Figs. 3. and 10). The markings did not appear on all of the cells and only occasionally were they sufficiently clear in the living cultures to be drawn with the camera lucida. When the cells began to spread out and migrate from the cut surface of the embryo, a few markings could be seen in the flat clear portion of the cell (text-fig. 1) and, as this region increased in extent, more striations became visible. The markings appeared to be on the under surface of the cells, that is, farthest from the mesenchyme. The striæ were very definite, appearing as longer or shorter dark lines over the cell, varying only slightly in width, and never crossing a cell boundary. The wall between adjacent cells was distinctly double, so that each cell had its own complete investment. This was also clearly seen in a few cases where ectoderm cells had become separated from each other. Frequently one or more striæ near the periphery of a cell were situated parallel with the cell wall, while the inner ones were more irregular or arranged concentrically with reference to several points in the cell.

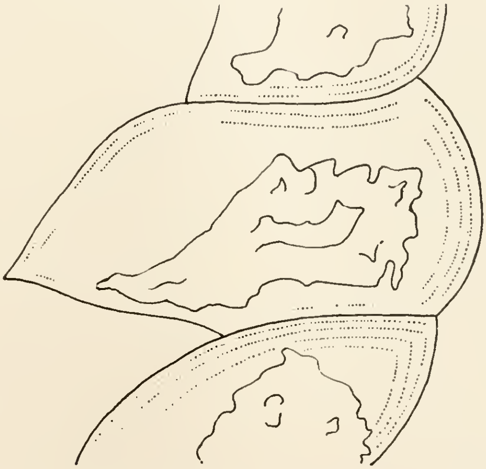

FIG. 1. Camera lucida drawing of an ectodermal cell in process of flattening out during migration; the central mass of thickened cytoplasm bordered by a clear flat region with pale concentric markings. Ocular 5, lens 4 mm.

Form and Behavior of Mesenchyme Cells.—The mesenchyme cells grew out from the explant as more or less elongated and separate cells, which later became connected in various ways. Adjacent cells sometimes sent out short broad processes along their sides, which appeared to fuse with each other, forming a reticulum with relatively small intercellular spaces (Fig. 9). Characteristic also of these cells was the projection of their protoplasm into short thread-like processes often giving a prickly appearance to the edge. The prickly processes became converted into protoplasmic bridges connecting the cells. When these were present the tissue appeared somewhat similar to the mesothelial membrane described by Lewis and Lewis ('12) in tissue cultures of the chick, except that in fundulus the cell boundaries are much less distinct. Whether the cells actually fuse or not is a question which is extremely difficult to determine with certainty. When the cells are connected with each other by extremely elongated protoplasmic processes, as shown in Fig. 3, the intercellular spaces are large and a wide-meshed reticulum results. There is a gradual transition from a membranous to a reticular arrangement of cells as the distance from the explant increases.

The outward growth of these reticular cells appears to be an important factor in the extension of the ectoderm. As previously noted, reticular mesenchyme cells were usually seen projecting beyond the ectodermal membrane (Figs. 1 and 3). These cells underwent very marked changes in form, their protoplasm flowing in a direction away from the ectoderm, the proximal portions of the cells, however, being connected directly or by intervening cells with the thickened edge of the ectoderm. The distal portions of the cells frequently developed broad fan-like expansions, which were firmly anchored to the cover glass.

The mode of formation of the fan-like expansions appears to be about as follows: The pseudopodia flow out from the cell in the form of delicate, radiating, thread-like or finger-like processes; then the region between the processes gradually fills out until a fan-like form is attained. This soon becomes entirely homogeneous near the periphery and adheres closely to the cover glass. Frequently the fan has a slightly fluted appearance as if not adhering equally well at all points.

As the mesenchyme cells migrate outward they exert a pull upon the edge of the ectoderm which is drawn outward, not by amœboid processes of its own, but by amœboid processes and fan-like expansions of the mesenchyme cells to which it is attached. The sheet of ectoderm is thus anchored in all directions as if by minute guy-ropes. When these are pulled more in one direction, folds and wrinkles are formed, which become smoothed out when the pull is equalized.

Pigment Cells.—Chromatophores of fundulus are large cells containing either black, brownish red, or yellow pigment granules. The cells migrate readily from the explant, but do not as a rule travel far. Stockard ('15) showed that the chromatophores have an affinity for plasma-filled spaces, being found adhering to the pericardium in normal embryos and to the heart itself in embryos deprived of a circulation. In the cultures of fundulus brown chromatophores were several times observed closely adhering to the beating heart, which happened to be projecting out into the fluid medium. Here they remained elongated for several days until the cultures degenerated. Isolated pigment cells that had wandered out upon the membrane degenerated more rapidly than other kinds of cells, and the pigment granules, freed from the cells, were readily taken up by the mesenchymal cells where they became aggregated around the nucleus (Fig. 6).

In addition to the pigment cells and those forming a membrane or reticulum other types of mesenchyme cells were observed. Among these were certain denser, more granular and vacuolated cells which migrated out upon the mesenchymal reticulum, and other cells (probably clasmatocytes) with brighter, more solid-looking protoplasm and numerous curved, finger-like pseudopodia. These cells contained numerous granules often derived from degenerated chromatophores. Brownian movement of these ingested granules was frequently observed.

Yolk Cells.—In some explants certain peculiar spherical cells filled with numerous clear greenish yolk spheres were seen massed together in the anterior part of the digestive cavity. These cells migrated readily out upon the mesenchymal reticulum where they became very slowly amœboid and wandered out along the edge of the ectoderm. Fig. 7 shows a group of these cells with the yolk spheres stained deep black with iron hæmatoxylin.

Cell Division.—The appearance of new cell boundaries in the ectoderm was frequently observed but in no case was a cell seen to divide. There is therefore no evidence as to whether the cells divide by mitosis or amitosis. In over fifty cultures stained with iron hæmatoxylin no stages of mitosis were observed. Two nuclei, however, were common in ectoderm cells (Fig. 8), as were also nuclei partly constricted into two, or with several irregular constrictions and variable nucleoli. There is no reason to believe that this condition is followed by division of the cells. It may be that the nuclei fuse together again, as Macklin ('16) observed in tissue cultures of the chick, and that the cells subsequently divide by mitosis. Holmes ('13) observed amitosis of the nuclei in tissue cultures of various tadpoles, but he states that nuclear division was not followed by division of the cytoplasm.

A few mesenchyme cells were observed to divide by mitosis and several groups of chromosomes in metaphase appeared in the stained material. Bi-lobed and double nuclei, indicative of amitosis, were also observed (Fig. 9).

Cultures of Chick and Fundulus.—A piece of fundulus tissue and a piece of muscle tissue from a chick embryo of eight days' incubation were placed together in a drop of Locke's solution containing chicken bouillon, and kept at 39° C. At the end of 48 hours each piece showed its characteristic form of growth, the fundulus having the double membrane previously described, the chick tissue showing the usual radiating type of outgrowth. In one region the fundulus outgrowth could be seen growing over a portion of the chick explant as over a foreign body. In another region outgrowths from the two pieces were almost in contact, but the cells from the two explants showed no tendency to intermingle. Specific differences were observable within the cells. Mitochondria are much more abundant in the chick tissues and the cytoplasm appears to be different, as shown by the greater ease with which the cellular structures of the chick may be observed.

General Considerations on the Movements of Membrane Cells.—The behavior of the cells in the cultures at different times is of considerable interest. Taking the normal form of the ectoderm cells as a standard, we find that the cohesive property of these cells is increased suddenly at the time the cut is made, as shown by the rounding up of the cells. The stimulus of the injury was sometimes effective for several hours. This influence seemed to be gradually weakened and the adhesive power progressively increased as the cells spread out in a thin layer upon the under surface of the mesenchyme. Holmes ('13) found that ectoderm cells of tadpoles in tissue cultures attach themselves readily to various kinds of substrata, including the cover glass, and "extend upon one another in mutual attraction which tends to keep them in continuous masses." In fundulus, however, this stereotropic activity of the ectoderm is called forth only when the cells are associated with the mesenchyme, and it appears to be much stronger than in the frog, causing the cells to be spread out in a thin single-layered membrane. In over fifty cultures it was never observed that an ectodermal membrane grew out unaccompanied by mesenchyme, whereas numerous cultures contained growths consisting of mesenchyme alone. The latter cells appear to be more highly stereotropic than the ectoderm, for they will adhere to the smooth surface of the cover glass even to the extent of having their processes snapped off when the ectoderm retracts.

The difference in behavior of the two layers of cells is perhaps correlated with the fact that under normal conditions of development ectoderm cells grow only in contact with the mesenchyme, whereas mesenchyme cells can grow in contact with widely varying kinds of surfaces.

The question arises whether there is any relation between wound-healing and the formation of the ectodermal membrane in tissue cultures. Loeb ('20) has discussed various processes involved in cell movements in wound-healing, designating among others amœboid migration of ectoderm cells, this being "the first response of the tissue to the wound stimulus." A factor in this amœboid wandering of the ectoderm cells is their stereotropic reaction, as expressed by their contact with the coagulum, which is "the foundation for the process of wound healing." In fundulus cultures the wandering of the cells in contact not with a coagulum but in this case with the mesenchyme layer is the foundation for the process of formation of the ectodermal membrane. The manner of cell movement, however, does not appear to be amœboid in character.

Oppel ('13) has described the bending of the skin edges along the cut surface of explanted pieces of the tadpole's tail as due to real movement; not simply a mechanical process but a change in form by which the ectoderm grows around the cut. In an earlier paper ('12*a*) he compares the ectoderm cells to partly filled sacs of inelastic material which can change their outline without varying the extent of their surface. This kind of movement is strikingly similar to the early changes which take place when the rounded cells begin to flatten. Oppel ('12*b*) distinguishes between epithelial movement, which is a mass movement, and amœboid motion which tends to isolate cells, as in connective tissue. He concludes that while the movement of the epithelium depends on the activities of the cells themselves, it is not an amœboid motion. The observations on fundulus confirm this interpretation. Here epithelial movement appears to be a mass movement throughout all stages in the formation of the ectodermal membrane. Single functional epithelial cells are never found.

In conclusion it may be stated that the activities of the cells in the formation of the ectodermal membrane in fundulus are similar to activities displayed also by ectoderm cells in the process of wound-healing. As in the latter, the cells exhibit mass movements the end result of which is to cover the connective tissue; so in tissue cultures of fundulus the migration of the ectoderm cells proceeds by mass movement which results in a partial covering of the mesenchyme layer. The contact reaction toward underlying connective tissue, exhibited by ectoderm cells in wound-healing, is paralleled by the stereotropic activity of the ectoderm cells evoked by contact with the mesenchyme. It may be said that the cells are attempting to follow out their normal activities, although subjected to abnormal conditions.

Summary.

1. Tissues of *Fundulus heteroclitus* grew in fluid media under conditions varying widely in respect to temperature, concentration of salts, and character of nutritive substances.

2. Mesenchyme cells migrated out into the medium upon the under surface of the cover-glass and formed almost continuous

or reticular membranes with isolated cells lying beyond. The ectoderm formed a membrane in close contact with the under surface of the mesenchyme. Nerve fibers, pigment cells, and yolk cells from the digestive tract readily migrated out. Peristalsis of the intestine, beating of the heart, contraction of the trunk musculature, and movements of the fins, were observed in numerous cultures several days old.

3. Characteristic of the ectoderm cells were certain delicate striations somewhat concentrically arranged, which formed an intricate pattern over the cell.

4. The mesenchyme cells are highly amœboid and possess characteristic fan-like expansions by means of which they adhere to the cover-glass and to each other.

5. While the initial stages in the formation of the ectodermal membrane were accomplished by migration and flattening out upon the under surface of the mesenchyme of the cells originally covering the body, the further extension of the membrane involved the formation and growth of new cells and tension exerted by the mesenchyme upon the thick edge of the ectoderm.

6. Mitosis was observed in several mesenchyme cells but not in the ectoderm, although new cell boundaries appeared from time to time. Frequently ectoderm cells contained two nuclei or one irregularly lobed nucleus.

7. During all stages in the formation of the ectodermal membrane the movement of the cells is a mass movement. Their reactions are much slower than those of the mesenchyme, and are never amœboid in character.

8. There is an essential similarity in the outgrowth of the ectodermal membrane and the process of wound-healing in respect to (*a*) the mass migration of the ectoderm cells, and (*b*) the stereotropic activity of the cells which is evoked by contact with the mesenchyme.

I wish to acknowledge my indebtedness, for the valuable suggestions and criticism, to Prof. W. H. Lewis and Mrs. Lewis, of the Department of Embryology of the Carnegie Institution of Washington, under whose direction this work was accomplished.

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DESCRIPTION OF PLATES.

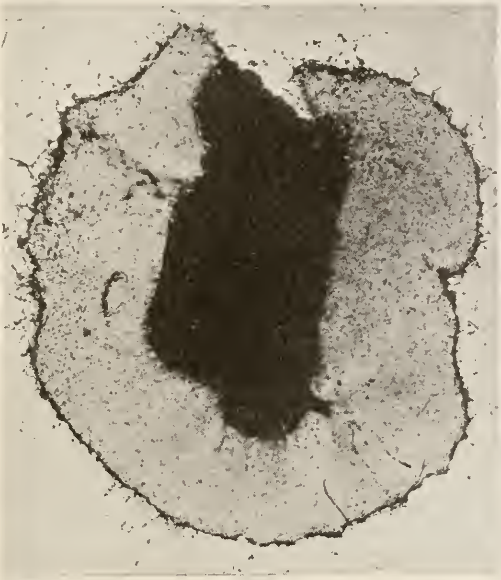
Figs. 1–10 are from photographs of cultures fixed in Zenker's solution without acid, and stained in iron hematoxylin. All figures except 1 and 5 were made with the high power. 1 and 5 were photographed with No. 4 Oc. and No. 16 lens.

Plate I.

Fig. 1. Seven-day culture from 15-day embryo, showing extent of double membrane composed of ectoderm and mesenchyme. The latter projects beyond the thickened edge of the ectoderm in the form of a loose reticulum or of isolated cells.

Fig. 2. Portion of outgrowth from an explant showing a continuous ectodermal membrane of large flat cells, and an imperfect membrane of darker granular mesenchyme cells, which lies above it.

Fig. 3. Portion of a reticulum of elongated mesenchyme cells, attached at one end to the thickened edge of the ectoderm, anchored at the other to the cover-glass by broad fan-shaped expansions. In some of the ectoderm cells delicate striations are visible.



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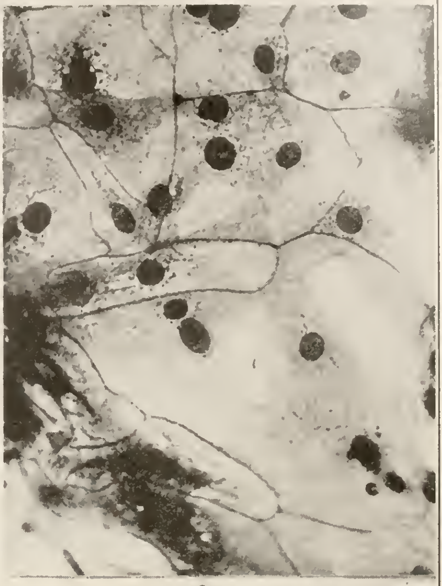

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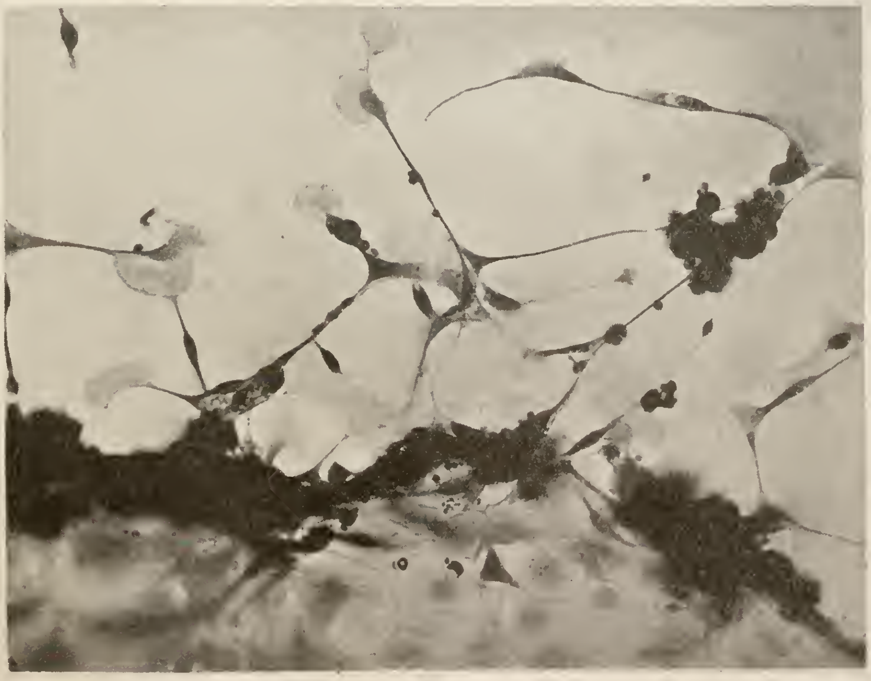

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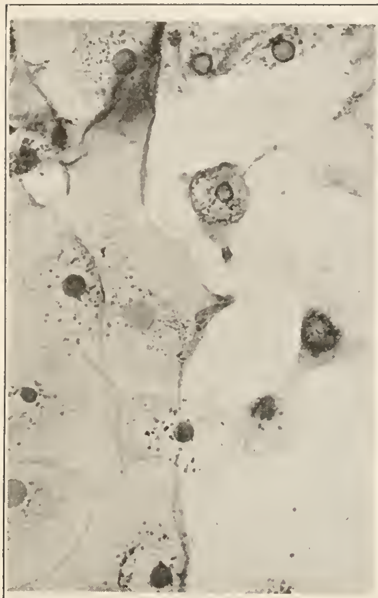
PLATE II.

FIG. 4. Portion of edge of ectodermal membrane, the cells almost completely flattened and slightly curled back at their extreme edge; mesenchyme cells projecting beyond them.

FIG. 5. Result of contraction of the ectoderm due to mechanical disturbance. The dark mass at the left is a retracted membrane of ectoderm and mesenchyme. Other portions of the mesenchyme remained adhering firmly to the cover-glass.

FIG. 6. Group of ectoderm cells, with large nuclei and faintly granular cytoplasm. Nuclei of mesenchyme cells are smaller, and are surrounded by dark granules derived from degenerated pigment cells; the boundaries of mesenchyme cells are not visible.

FIG. 7. Group of yolk cells lying upon the mesenchymal membrane where they had migrated from the digestive tract. The yolk spheres are stained deep black with hematoxylin.



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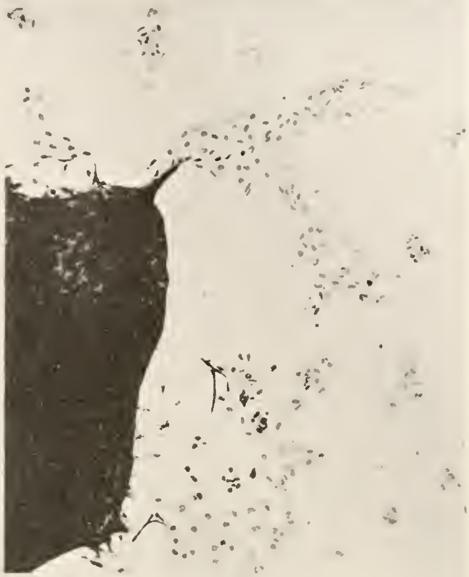

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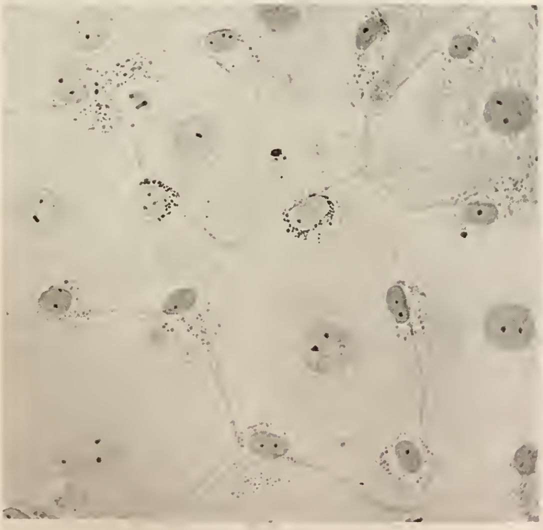

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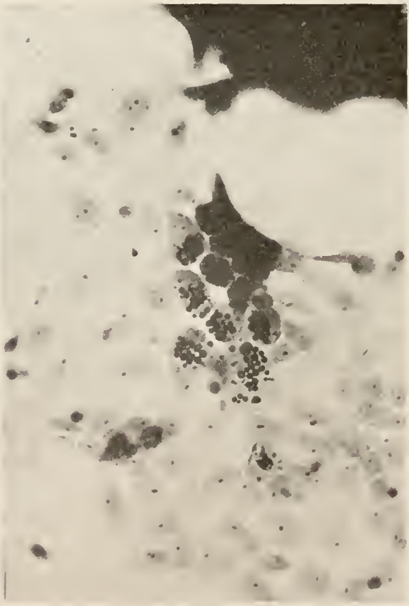

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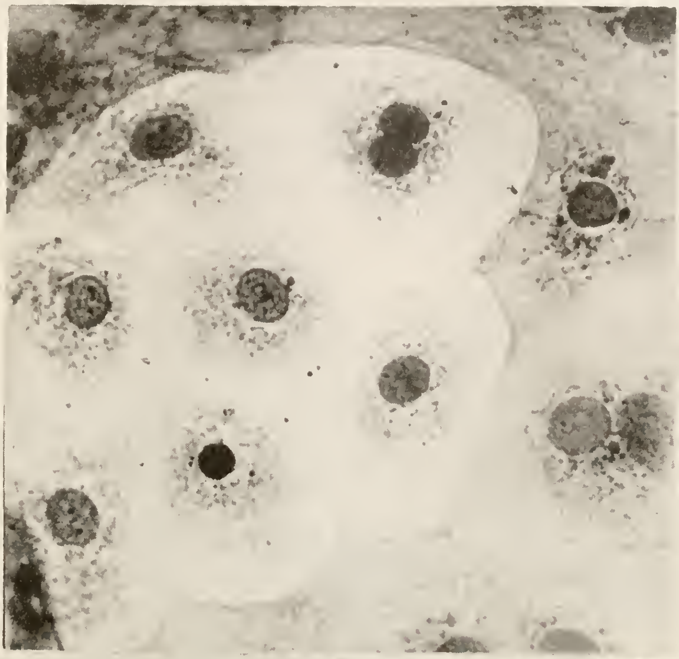
PLATE III.

FIG. 8. Group of seven ectoderm cells, free from overlying mesenchyme. A bilobed nucleus is visible in one cell.

FIG. 9. Group of mesenchyme cells forming a membrane with relatively small intercellular spaces. Mitochondria are visible in the form of threads and granules.

FIG. 10. Portion of outgrowth showing striations in ectoderm cells. Nuclei and cytoplasm of mesenchyme cells are visible as dark masses upon the ectoderm.



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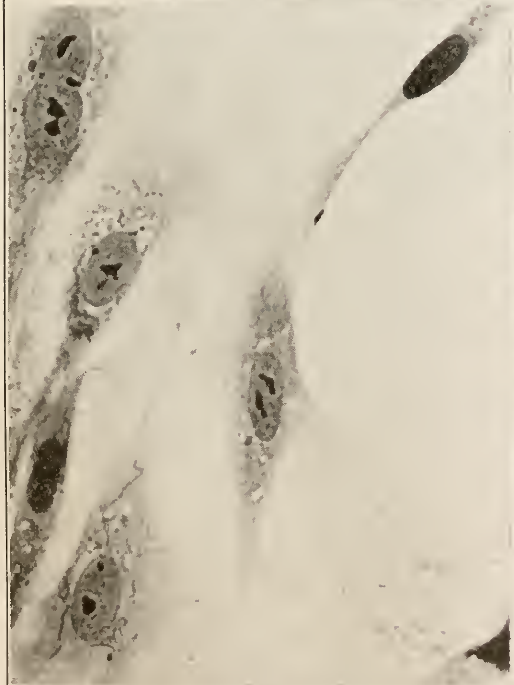

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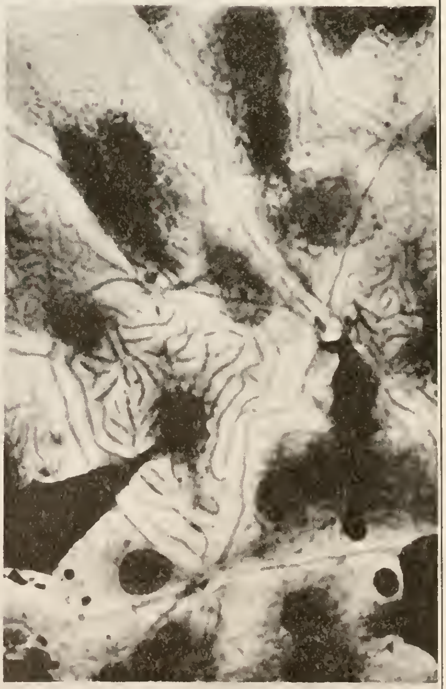

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THE PRESENCE OF GLYCOGEN IN THE CELLS OF EMBRYOS OF FUNDULUS HETEROCLITUS STUDIED IN TISSUE CULTURES.

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At the present time it is practically impossible to demonstrate the various chemical substances of the living cell, owing to the fact that most means of analysis cause the death of the cell. A few of the differential stains, such as Sudan III. and Nile blue, have been applied to studies of living tissues, but these do not behave in the same manner in the living cell as in dead material (Lewis and Lewis, 1915; M. R. Lewis, 1918). In most cases the chemical nature of cytoplasm has been discussed from the standpoint of results obtained from dead cells, and it is doubtful whether such conclusions can be applied directly to the living. This is true in regard to the experiments given herein; for, while the substance described reacts as does glycogen to the tests usually employed to demonstrate glycogen, these results were not obtained until after the cells had begun to be affected by the iodine.

Bernard (1859) demonstrated the presence of glycogen in many kinds of tissues and since that time several methods for showing this substance in the cell have been reported. The methods most frequently used for histological purposes are those of Best (1909), Gage (1917). Neither of these was used in the experiments given below. Instead, the living cells were exposed to iodine vapor in such a manner that they could be followed throughout the experiment, *i.e.*, when living, while dying, and after death had occurred.

TECHNIQUE.

Small pieces of fundulus embryos (just before hatching) were explanted into hanging drops of the following solution: 80 c.c.

diluted (40%) sea water plus 0.02 per cent. $NaHCO_3$ plus 20 c.c. fundulus bouillon plus 0.5 per cent. dextrose (M. R. Lewis, 1917). Within 24 to 48 hours large growths were present around these explants. The cells composing the growths were then exposed to iodine vapor by scattering a few fragments of an iodine crystal in the bottom of the hollow ground slide under the hanging drop, care being taken to prevent them from touching the drop. The vapor from the iodine crystal penetrated the hanging drop and acted upon the cells. Just a sufficient amount of iodine should be used to rapidly color the cytoplasm yellow and to show the port-wine color of the glycogen within one to two minutes.

NORMAL FUNDULUS CULTURES.

As has been shown by Dederer (1921), the ectoderm and the mesenchyme cells grow out from explants of fundulus embryos in the form of membranes. The mesenchyme cells are usually attached to the cover-slip and the ectoderm cells form a layer directly beneath them. Mesenchyme cells extend beyond the ectoderm and also along the edge of the membrane and those scattered farther out on the cover-slip are the only cells which have processes to any extent. These processes usually spread out at one end into a large, thin, fan-like structure. One or more large, flat, oval cells, probably endoderm, are sometimes found on the membranes; these are quite different from the mesenchyme cells, in that they have an oval shape while the mesenchyme cells are elongated or somewhat hexagonal, and their cytoplasm also appears to be of a different consistency. For convenience of description these cells will be called *oval* cells. When the cultures were placed over fragments of an iodine crystal the cytoplasm and nucleus of all the cells became yellow, the the mitochondria a darker yellow, and the fat globules a brownish color. Almost the entire cytoplasm of the oval cells, regions of the cytoplasm of some of the mesenchyme cells, and certain parts of a number of their processes turned a port-wine color. This was a distinctly different tint from that exhibited by any other part of the growth and was the same as the characteristic color exhibited by glycogen when exposed to the action of iodine.

The three regions which showed this color reaction will be discussed separately.

1. *Oval Cells.*—As the iodine vapor penetrated the hanging drop, the cytoplasm of these cells became a pale yellow; then the granular portion, including the mitochondria and fat droplets, *i.e.*, the endoplasm, appeared to shrink slightly and became a deeper yellow. Meanwhile the remainder of the cytoplasm, except a thin yellow ectosarc, turned pink, the color gradually deepening until, after a few seconds, this portion was a deep port-wine color, while the endoplasm, nucleus and ectosarc remained yellow. At times the port-wine-colored material occupied the greater part of the cell, leaving only a small clump of endoplasm and the nucleus. In other cases the yellow endoplasm, nucleus and ectoplasm took up practically the entire cell except what appeared as a large port-wine-colored vacuole. The contour of the cell did not change but remained the same size as it was before exposure to the iodine. In a few of these cells there were a number of vacuoles, which also became port-wine color. The color was more intense in tone in the oval cells than in any other portion of the growth. It remained in them for some time, in a few instances for over an hour, then faded, and the whole preparation became a dark yellow color.

2. *Mesenchyme Cells of the Membrane.*—Of the cells forming the membrane probably only the mesenchyme exhibited the port-wine color upon exposure to iodine. This was difficult to determine definitely because the cells adhered together so closely, and also because of the fact that, while the mesenchyme cells were frequently found extended beyond the ectoderm cells, the latter were never observed separated from the former. Not all of the mesenchyme cells showed the port-wine color when in the presence of iodine, and which ones would do so could not be foretold. In these cells the cytoplasm of the central and thicker portion of the cell became a diffuse pink color, while the nucleus and ectoplasm became yellow. This area of pink coloration was not definitely limited but toned off into an extensive yellow ectoplasm. The arrangement of the mitochondria and other structures was the same in the cells having a pink area as in

normal cells. Gradually the pink color became deeper in tone until it attained a pale port-wine color. Within a few seconds the color disappeared from the central region of the cell and a large, round port-wine-colored bleb appeared at one side of the cell. Practically no change was observed in the structure of the cell during the disappearance of the stain and the formation of the bleb. Many of these blebs appeared scattered over the membrane, especially in the region adjoining the explanted piece. The color remained in them for about twenty minutes and then faded out, leaving the blebs rather undefined and difficult to distinguish. In some of the more peripheral cells of the membrane the port-wine color remained diffuse in the cytoplasm of the central portion for some time and then faded out without forming blebs. The mesenchyme cells which had migrated out on the cover-slip, away from the membrane, seldom formed blebs.

3. *Cell Processes.*—The processes of the cells formed the region where the appearance of the port-wine color could be observed most clearly. The large fan-like processes referred to above exhibited lighter and darker areas where the cytoplasm varied either in density or in thickness. After exposure to iodine some of the lighter regions became pink. Later these turned into distinct port-wine-colored areas in the yellow cytoplasm. A few of the processes did not exhibit these stained areas at all; in others some of the areas remained quite pale in color. This phenomenon did not continue for longer than half an hour; at the end of this time the color had faded and that area of the process was slightly shrunken.

When a drop of saliva was placed upon a culture which was later exposed to iodine vapor the port-wine color was not found in the cells. Death of the cell also prevented its appearance. No granules having the characteristic glycogen color were seen in any of the cultures. Neither the mitochondria nor any other granules were concerned in the formation of the port-wine-colored areas. It seemed as though the material which exhibited the typical port-wine color was diffuse throughout certain parts of the cytoplasm and became more definitely localized during the death of the cell, which occurred coincidentally with the iodine staining. Thus it is seen that some substance, which reacts as does glycogen when exposed to iodine, is present in the cells of fundulus cultures. It is possible that this substance is glycogen. That the substance which became port-wine colored was not the dextrose itself is shown by the fact that dextrose placed in Locke's solution did not so stain when exposed to iodine, while glycogen did.

A few cultures of chick embryos were tested in the same manner for a comparison with those of the fish embryos. No port-wine color was observed in the cells of the older embryos except in one somewhat degenerate culture where a few blebs were already present on certain of the dying cells; these blebs became slightly pink but in no case was there the port-wine coloration such as occurs in fundulus cultures. On the other hand the cells in cultures of very young embryos (48 hours) sometimes contained an abundance of this substance.

The Influence of Starch Upon the Amount of Glycogen Present in the Cells.

Soluble starch (Kaulbaum) was added to the medium of the cultures of fundulus embryos in order to determine whether it could be utilized by the cells to store up glycogen. The starch was dissolved in distilled water and boiled for two minutes; 60 c.c. of the starch solution was then added to 40 c.c. of sea water and the medium prepared in the same manner as for normal cultures. When the quantity of starch was less than 0.1 per cent. it had no appreciable effect upon the cells. In these cultures the growth was normal and no increase in the amount of glycogen could be detected. When larger amounts of starch were added, or when the starch became slightly clumped into masses of very small granules, as sometimes happened, small particles were occasionally taken up by the cell and appeared within the cytoplasm as small granules or granular masses, in some cases surrounded by a vacuole. Upon exposure to iodine the starch became blue, whether within the cell or in the medium. The surrounding vacuole became pale blue, or sometimes lilac, but never the port-wine color indicative of glycogen. The port-wine color was present in the same regions as in normal cultures, but never greater in amount and sometimes less than in the normal control preparations. Even after a number of days the starch did not become changed into glycogen as, for instance, one 8-day-old culture in 0.75 per cent. starch solution, exposed to iodine, exhibited cells in which there were a few vacuoles some of which contained blue granules. The vacuoles were pale blue or lilac but never port-wine color.

Influence of Dextrose in the Medium Upon the Amount of Glycogen in the Cells.

Cultures were prepared in a medium free from dextrose in order to ascertain whether the lack of dextrose would prevent the appearance of glycogen in the cytoplasm of the cells. The results from twenty such cultures show that, while the amount of glycogen could be decreased by the lack of dextrose in the medium, its presence could not be entirely inhibited. Some of these cultures (48 to 72 hours) exhibited only a slight trace, if any, of the port-wine color when exposed to iodine. On the other hand, a few did contain decided evidences of a small quantity of this substance.

Explants into media containing 0.5 per cent., 1 per cent. and 2 per cent. dextrose, made at the same time as those without dextrose, showed a decided increase in the quantity of glycogen up to a certain point. In no instance did all of the cells of a culture exhibit the port-wine color. Neither did any one cell become greatly filled with this substance. Of all the cultures, those grown in a solution containing 2 per cent. dextrose exhibited the most marked amount of the port-wine-colored material; that is, more cells contained this substance, practically all of the fan-shaped processes had regions which were stained port-wine color, and the color was deeper in tone and did not fade as rapidly as in the normal cultures. Saliva placed upon cultures in 2 per cent. dextrose prevented the appearance of the port-wine color, just as it did in the normal cultures. In these experiments with different amounts of dextrose it was impossible to predict whether a given cell would show the port-wine color in the presence of iodine. The cytoplasm was not characterized by any structure indicative of this substance, but appeared the same in all of the cells. The processes of the mesenchyme cells had the peculiar lighter areas which in some cases became port-wine colored and in others remained pale yellow.

Summary.

The cells of *Fundulus heteroclitus* grown in tissue cultures contain some substance which behaves in the same manner as does glycogen in the presence of iodine. It is possible that this substance may be glycogen.

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BIOLOGICAL BULLETIN

ON THE INFLUENCE OF TEMPERATURE ON THE EXCRETION OF THE HIBERNATING FROG, RANA VIRESCENS KALM.

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Almost all vital phenomena have been studied as to the influence of temperature on their magnitude. In his excellent book on "Temperatur und Lebensvorgänge,"[2] Kanitz gives a summary of the work done in this field, and tries to show that the R-G-T rule of van't Hoff-Cohen holds true also for the reactions of organized material. In Loeb's laboratory it was found by Snyder and Robertson that the heart-beat of invertebrates and of the lower vertebrate follow this rule. These observations have been confirmed and amplified by several authors. For the mammalian heart also some investigators—among them Snyder—tried to show that its frequency follows van't Hoff's rule. Other observers however could not agree on this point and emphasized the complexity of the phenomenon, which would make it improbable that this rule, which holds true for simple chemical reactions, could be applied to these more complex phenomena. The rhythm of breathing, the velocity of conduction in the nerve, the activity of the muscles, the geotropic and phototropic movements of plants, the effect of poisons, the length of life, the rate of development and growth, all have been studied from this point of view, and a great diversity of opinions exists as to whether the rule of van't Hoff holds true or not.

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[1] Thanks are due to Mr. R. Goldberg, who made some of the total nitrogen, urea and ammonia determinations for me.

[2] Aristides Kanitz, "Temperatur und Lebensvorgänge," *Berlin. Gebr. Borntraeger*, 1915.

The advocates of the confirming answer (as for instance Snyder and Kanitz) explain the little differences between the theoretical values and the experimental results as due to errors in our methods, while those who deny the parallelism between the reactions of organic material and those of ordinary chemistry emphasize these differences and sometimes claim a regular decrease in the value of the temperature coefficient, as Krogh and Ege did in a controversy with Snyder.

Several authors studied the influence of temperature on metabolic processes. The CO_2 assimilation of plants was shown by Matthaei and numerous other authors to follow the rule of van't Hoff. The frog's metabolism was first studied by Hugo Schulz.[3] The principal importance of his work was that he showed that temperature has a tremendous influence on the frog's metabolism. The frog's output of CO_2 is according to him more than 16 times as much at 25° as at 0°. Aubert[4] gave more accurate figures, but they can still not yet be used for checking them up with the formula of van't Hoff. Vernon's publications,[5] in which he tried to show that between 2° and 17° the CO_2 output was constant, provoked some other papers; the results seem to be due to the sudden changes in temperature to which he subjected the animals. The same fact, a very slow increase in metabolism between 10° and 20°, has also been found in *Cyclodus gigas*, a lizard, by C. J. Martin[6] and in the work of some other investigators.

EXPERIMENTS.

In my own experiments I tried to determine the influence of temperature on the excretion of winter frogs. Ten animals were kept for 24 hours in a small aquarium. The bottom was covered with some distilled water; care was taken to keep this quantity as constant as possible for reasons given in a previous paper.[7] By taking 10 animals at the same time the individual differences were eliminated as much as possible. The aquarium was placed in a larger water container in which the temperature of the water could be automatically regulated, whereas a stirrer moved by a motor kept the water constantly in motion. After 24 hours the urine was centrifuged—to remove the skin particles and the fæces—and then measured. In this urine I ran total nitrogen, urea, ammonia and uric acid determinations.[8] A difference of ten degrees was chosen because the classical formula of van't Hoff's rule speaks of 10°. Higher temperatures than 31° could not be used because a temperature of about 33° is fatal for the frog as appeared in some experiments in which I found all animals dead after having them kept for some time at higher temperatures. Even in the 31° experiments some of the frogs were very faint and near death after 24 hours.[9] This is probably the reason why my figures for this temperature were much more irregular than the rest—in one of the experiments I got for instance 70 mgm. total nitrogen. The results are given in Table I.

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[3] Hugo Schulz, "Ueber das Abhängigkeitsverhältnis zwischen Stoffwechsel und Körpertemperatur bei den Amphibien," *Pflüger's Arch.*, 14, 78–91, 1877.

[4] Hermann Aubert, "Ueber den Einflusz der Temperatur auf die Kohlensäure Ausscheidung und Lebensfähigkeit der Frösche in sauerstoffloser Luft," *Pflüger's Arch.*, 26, 293–323, 1881.

[5] H. M. Vernon, "The Relation of the Respiratory Exchange of Cold-blooded Animals to Temperature," *Journ of Physiol.*, 17, 277–292, 1895. H. M. Vernon, "The Relation, etc., Part II," *Journ. of Physiol.*, 21, 442–496, 1897.

[6] C. J. Martin, "Thermal Adjustment and Respiratory Exchange on Monotremes and Marsupials," *Transact. Roy. Soc. London*, (B), 195, 1–37, 1902.

TABLE I.

Temperature.	Total Nitrogen.	Urea and Ammonia Nitrogen.	Urea.	Ammonia Nitrogen.	Ammonia.	Uric Acid.	Nitrogen in it.
1°	6.55	6.5	10.5	1.6	1.9	0.0	0.0
11°	10.45	9.2	13.8	2.8	3.4	0.13	0.04
21°	16.3	14.6	23.9	3.5	4.2	0.30	0.10
31°	59.03	50.8	60.4	22.7	27.6	0.83	0.28

[7] H. C. van der Heyde, "Studies in Organic Regulation, I., The Excretion and the Blood-Picture of the Hibernating Frog," *Journ. Biol. Chem.*, XLVI., 1921, p. 421.

[8] The total nitrogen, urea and ammonia figures are the average of three series of determinations on each of which the determinations were made *in duplo*. It should be noted that the figures of one series were not identical with those of the others; but that though the way of increase of each series was identical the absolute values showed some variation. The uric acid figures have only been determined in one series. As previously I wish to state that my trust in the uric acid figures is not very great for reasons given in my previous paper (7).

[9] It seems that the highest temperature which *Rana pipiens* tolerates according to the experiments of Cameron and Brownlee (*Transact. of the Royal Soc. of Canada*, Ser. III., Vol. IX., p. 67) is even lower.

The figures for the total nitrogen are graphically represented in Fig. 1.

It is clear that temperature has in reality a tremendous influence on the frog's catabolism. From 0° till about 20° this increase is only relatively slight. After 20° however the curve rises almost vertically. When we compare our curve with that given by Lusk[10] after the figures of H. Schultze, we notice a striking similarity between the two curves. It is clear moreover that this fact has a great biological importance. The frog in his hibernating stage at 4° in the mud can not have the intensive metabolism which the frog in midsummer "as he sits on the

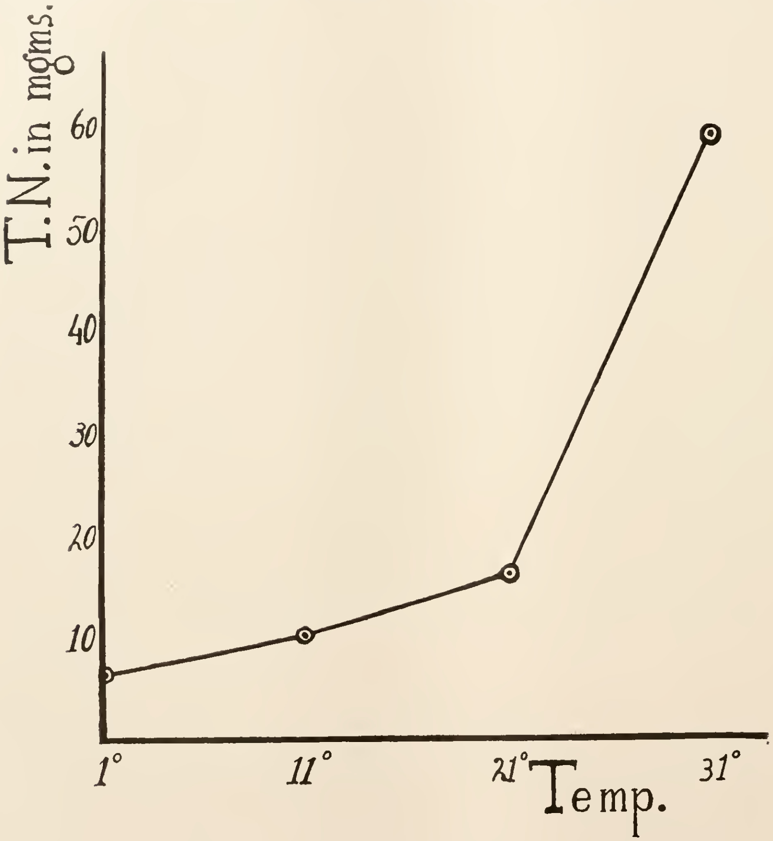

FIG. 1. The influence of temperature on the total nitrogen excretion.

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[10] Graham Lusk, "The Elements of the Science of Nutrition, "3d edition, W. B. Saunders Co., Philadelphia and London, p. 115.

river bank and snaps at passing flies" sustains. The change in temperature of its blood causes its tissues to show a much more intensive metabolism. Krogh in his numerous studies on the influence of temperature on poikilothermous animals gave as his opinion that the influence of temperature on the animal's metabolism is of double nature. On the one hand the basal metabolism is increased, on the other hand the tonus of the muscle is increased and causes in that way an increased muscle metabolism. The latter process is regulated by the central nervous system and in fact he observed that in decerebrated animals the temperature did not have as much influence on the CO_2 output as in normal animals. Moreover Krogh is of the opinion that a regular decrease can be observed in the temperature coefficient. For this reason I figured out the temperature coefficients of my own experiments which are given in Table II.

Table II.

Range.	Coeff.
1–11°	1.6
11–21°	1.56
21–31°	3.6

Instead of a decrease we notice an increase. We must however keep in mind that these experiments have been made on normal animals. The quoted experiments of H. Schultze give the same result.

I do not dare to say in how far the rule of van't Hoff holds true for this case. I believe that we can not be careful enough in drawing conclusions on this point. The reactions of the organism as a whole can not but with extreme care be compared with simple chemical reactions. Not only the two factors emphasized by Krogh play a rôle in the processes of which we see the final result in our urinary analysis, but also the blood pressure, the water intake which has been shown by Overton[11] to be very strongly influenced by temperature, and the function of the kidneys. For this reason it seems not very probable that the final result of all these processes should be comparable to a simple chemical reaction. When however we calculate the temperature coefficients we see that they are not constant enough to give us the right to the conclusion that van't Hoff's rule holds true in this case, but on the other hand they do not prove the contrary.

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[11] E. Overton, "Neununddreiszig Thesen über die Wasserökonomie und die osmotischen Eigenschaften der Amphibienhaut," *Vorl. Mitt. physik. medizin. Gesellsch.*, Würzburg, N. F., 36, 282, 1904.

To one remarkable phenomenon which I observed in my experiments I might still draw attention. As stated in my previous paper pure frog urine as obtained from frogs in the vivarium in which the average temperature is below 10° is absolutely waterclear. Toda and Taguchi however in their paper on the inorganic constituents of the frog's urine observed a slight yellow color. Their experiments were made on summer frogs. Now it is very remarkable that indeed this pigment appeared in my experiments in which higher temperatures were used. Fig. 2 shows a picture of three samples obtained in three different experiments at 11°, 21° and 31°. The increase in color which was even more striking than this picture shows runs completely parallel with the nitrogen content as represented in Table I. and Fig. 1.

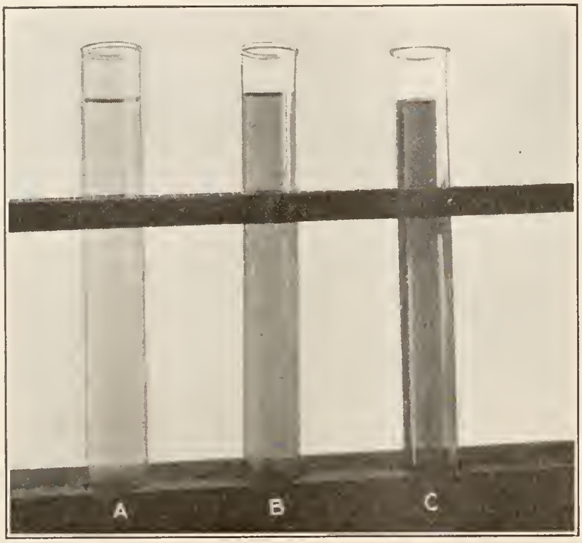

Fig. 2. Influence of temperature on the appearance of the yellow pigment of the frog's urine. *A*, 11°; *B*, 21°; *C*, 31°. As the color of the 1° urine did not differ from that of 11° only these three have been photographed.

Zusammenfassung.

Die Temperaturabhängigkeit der Stickstoffelimination von überwinternden Frösche (*R. virescens*) wurde studiert. Die Zahlen sind in Fig. 1 graphisch dargestellt. Ein gelbes Pigment tritt auf wenn höhere Temperaturen benutzt werden, die Intensität der Färbung geht dem Stickstoffgehalt und der Temperatur parallel (Fig. 2).

NOTE ON THE PIGMENT OF ARBACIA EGG-SECRETION.

OTTO GLASER.

I.

If the unfertilized eggs of *Arbacia punctulata* are allowed to secrete into a small volume of sea-water, the latter, in the course of five or ten minutes, takes on an amber color. The density of the pigment varies with the concentration of the eggs as well as with the time during which they secrete. After an hour, the sea-water is apt to become reddish-brown.

The later discoloration is due, at least in part, to the elimination of echinochrome—a substance which these eggs contain in considerable quantity. Whether the pigment given off during the earlier moments of secretion is also echinochrome remains uncertain since the diagnostic reactions recommended by MacMunn ('85) are not convincing at the dilutions at which it is necessary to work. In exudate of more than "standard" strength I have been unable to see that HCl produces a red tint or that NaOH intensifies the yellow. With precipitated pigment both acid and alkali seem to intensify the yellow color to an extent barely perceptible.

II.

One cannot assume, offhand, that the pigment is either important or negligible in fertilization. In my study of egg-exudate, therefore, I first attempted to decolorize the secretion in order that the lipolysin and agglutinin subsequently recovered by other methods might be either free or relatively free from pigment. For this purpose charcoal cannot be used since it has the serious disadvantage of removing the whole, practically, of the organic reaction system. Moreover, the pigment, if wanted for separate study, cannot be recovered readily from the charcoal.

To isolate the pigment, it is much better to use chloroform as a decolorant. The method is very simple. To a given volume of fresh exudate, one adds, roughly, half a volume of chloroform and shakes vigorously for fifteen minutes. In this time an emulsion is formed in which the individual globules are remarkably stable. The system, indeed, is a jelly, white in appearance and surprisingly voluminous. In fact, both its volume and stability at first misled me into thinking that I had found a method for precipitating, if not all, at least the greater part of the organic solutes present.

III.

On standing, the jelly separates from both the unemulsified chloroform and the remainder of the exudate.

The degree to which the latter is decolorized varies, among other things, with its age. If the exudate is perfectly fresh, the pigment is removed almost if not quite completely; if the secretion is 36 or more hours old, decolorization is more difficult and the chloroform jelly less stable.

Microscopic examination of the jelly reveals on the surface of each chloroform globule a delicate skin, translucent, with pearly sheen, continuous, yet also with suggestions of extremely fine fibrils. As the chloroform evaporates, this skin wrinkles until finally there remains an empty bag.

Since the original exudate contained sea-salts, I washed the jelly on a filter or shook it for half an hour in several changes, first of fresh, and later of distilled, water. Under this treatment the globules of chloroform break up into still smaller spheres greatly increasing the stability of the system. The jelly can be freed from sea-salts entirely and in this state has been kept for weeks in stoppered bottles.

IV.

The material in the walls of the globules can be recovered simply by permitting the chlorofrom to evaporate. Slight heat naturally facilitates the process. The jelly, also, may be broken down instantaneously by means of 95 per cent. alcohol. The vesicles are permeable for the alchohol and this, itself, is soluble in chloroform. Since the material held in the walls of the globules is insoluble in both alcohol and chloroform, precipitation is inevitable.

Macroscopically, this precipitate appears to be coarsely flocculent. Its color is yellowish-brown. Under the microscope, granules aside, one sees chiefly fibers. These, when dried on a filter, yield thin felt-like sheets which cannot be readily dissolved in either sea-water or fresh. The material is only slightly soluble in acids and alkalis.

V.

The solubilities of the precipitated pigment are such that it is very difficult to test the importance of this material in fertilization. So far, nothing that would merit particular attention has come to light and the conclusion that properties highly significant in fertilization are absent is reinforced by the eggs of the starfish, the sand-dollar, the oyster, *Nereis* and *Fundulus*, none of which, apparently, secrete anything that corresponds at all closely with the *Arbacia* pigment. However, there is one suggestive fact: after removal of the pigment, the *Arbacia* exudate, physically, is a less stable system than before. From unmodified exudate nothing free from sea-salts can be precipitated with 95 per cent. alcohol; with the pigment removed, 95 per cent. alcohol, insufficient to precipitate the sea-salts, throws down the sperm-agglutinating material. It appears therefore as though the pigment in some way stabilized the exudate.

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Amherst College,
March 1, 1921.

SEX RATIOS IN FŒTAL CATTLE.

F. M. JEWELL.

I. Introduction.

It is a matter of common knowledge that among animals there is an approximate equality of numbers between the sexes, and the possible causes for this equality have long been a matter of speculative interest. Since the dimorphic character of the spermatozoa in various animals including cattle (Wodsedalek '20) has been confirmed by many investigators, it is generally conceded that the sex is determined as soon as the successful spermatozoön enters the ovum.

Therefore, according to the laws of chance, one would expect to find an equality of sexes at the time of conception. However, a vast amount of evidence shows that this is not the case, but that throughout the animal kingdom, with few exceptions, there is a preponderance of males at birth.

In the collection of twins in fœtal cattle used by Dr. F. R. Lillie in his work on the free-martin, the sex ratio was 134 males to 100 females and although the total number was but 108, this represented such a wide departure from the sex ratio at birth, as given by other investigators, as to demand attention. This indicates that there may be either a marked difference in the sex ratios in the fœtal and the born population, or that there is some interference in the chance determination of sexes in dizygotic twinning in cattle.

This investigation was undertaken to determine the fœtal sex ratio in cattle in order that this discrepancy might be cleared up, but more particularly to procure data for comparison of the primary, secondary and tertiary sex ratios as stated by A. M. Schultz ('18).

First it will be necessary to give a statement of the existing data on sex ratios in cattle, expressed as the number of males per 100 females.

Wilckens ('87) gives the ratio in cattle at or near the time of birth as 107.3, the number of individuals being 4,900. Pearl and Parshley ('13) in their studies on the sex ratio in cattle in relation to coitus and the period of œstrus give the ratio as 113.3 in a total population of 480. In more recent data by Pearl ('17) covering 1,313 individuals the sex ratio was 100.12.

It should be pointed out that in other animals the data shows that there is no correlation between the sex ratio and multiple births. Parker and Bullard ('14) and also Wentworth ('14) have shown this to be true for pigs; King and Stotensburg ('15) for rats, and Newcomb ('04) showed that in man the sex ratio in twins was practically the same as in single births.

It should be understood that a different rate of mortality in the sexes either during intrauterine development or after birth would cause the sex ratio to vary at different ages, and for this reason the sex ratio is usually spoken of as primary, secondary and tertiary. The primary is the ratio determined at conception and is the original sex ratio; the secondary is that at time of birth and the tertiary during adult life.

A. M. Schultz ('18) attempted to determine the primary sex ratio for man in an indirect way from the data on the mortality of embryos and fœtuses combined with the sex ratio at birth. In doing this he stated that only in case the mortality of the two sexes was equal would the primary and the secondary sex ratios be equal; that if the male and female abortions were absolutely equal, the sex ratio would be smaller at conception than the secondary, and that if there were a greater intrauerine mortality for males than for females, then the primary sex ratio would be greater than the secondary in proportion to the number of abortions and stillbirths.

In order to determine the primary sex ratio, he ascertained the sex ratio in abortions and stillbirths, and the number of such cases for every 100 living born, both male and female. From the data as given by various writers and from the material that he used (nearly 600 fœtuses of the embryology department of Carnegie Institution) he established the following probable values:

For each 100 living born with sex ratio of	105.5
8th to 10th month—4 stillborn, sex ratio of	130.0
4th to 7th month—9 abortions, sex ratio of	106.3
0 to 3d month—14 abortions, sex ratio of	125.0
Total conceptions, 127, sex ratio	"X"

Thus, for every 100 living born he concluded that there were 127 conceptions; 100 with a sex ratio of 105.5, and "a" stillbirths and abortions with a sex ratio "b," in all with a primary sex ratio "X." This primary sex ratio he found to be 108.47. Schultz also quotes the determination arrived at by other investigators as follows: Bernoulli, 108.2; Gendrassiks, 108.2; Lenhossek, 111; Auerbach, 116.4 (who believed that it would reach 125 if certain corrections could be made). Schulze thought that it would not exceed 110.

It was more especially in relation to the primary sex ratio and to determine whether there was a different viability in the male and female fœtuses in cattle that the present investigation was undertaken and with these facts as a basis the data obtained are presented. Special acknowledgment is due Dr. F. R. Lillie, who suggested the problem and gave valuable assistance in the interpretation of the data.

II. DATA.

A. *Method.*—The work in collecting was done at one of the large packing plants in Chicago during the spring of 1919. In butchering the cattle at the plant, every uterus containing an embryo is taken to a certain room and if the calf is large enough, the skin is saved. Thus the writer was able to open the uteri and record the data directly as each fœtus was removed. In this way any errors or neglect in birth registration are avoided.

The data embraces 1,000 individuals and the sex and length, as an indication of age, were recorded. Observations were also made on the number of corpora lutea in every case where this was possible, especially when twins were found, in which case the position in the uterus also was noted. The complete tabular list with the crown-rump measurement and sex of each individual as removed from the uterus is omitted in this article.

B. *Items of General Importance.*—In regard to the number of corpora lutea, in all of the ovaries examined, about 300 in all, they corresponded in number to the number of fœtuses with one exception. This exception was a pair of identical twins from one ovum, and since we are concerned with the sex ratio at the time of conception, obviously only one of these should be recorded in the data. In this case both ovaries were present, there was only one corpus luteum, and the twins of course were in one horn of the uterus and both of the same sex. This case of monozygotic twins is of very rare occurrence in cattle, being the first observed in the collection of 108 twins in the Zoölogy Department of the University of Chicago. There were four pairs of twins in the 1,000 fœtuses, and as is commonly the case in twins of opposite sex, the male is usually a little farther along in development than the female. In numbers 646 and 647 the male was 3.5 cm. longer than the female, the latter being 65.0 cm. long. In 807 and 808 the males were both the same length, 68.5 cm. In 988 and 989 the male was 6.1 cm. longer than the female, the latter being 58.2 cm. long.

Twins Numbers 648 and 647 and also 807 and 808 were in separate horns of the uterus and there was one corpus luteum in each ovary; while numbers 988 and 989 were in one horn of the uterus and there were two corpora lutea in the ovary of that side. Since the ovaries on some days were removed for commercial purposes before I had access to them, it was impossible to obtain data of this kind on the total of 1,000 fœtuses.

C. *Analysis of Data.*—Since the length of the embryo can be used as an indication of its age, we can arbitrarily make certain groupings and assume that those within that group are on the average at about the same age.

In Table I. is given such a grouping according to length, from 0–10 cm., 10–20 cm. etc., up to 90–100 cm. The smallest individual was 4.2 cm. in length and the largest was 95.3 cm. in length. Thus the data extends from a comparatively early period in fœtal development practically to the time of birth.

The lengths of embryos were tabulated in lots of 50 in order to get some idea of how the sex ratio would vary according to the position of the group in the total of 1,000 fœtuses. Thus in the first space in Table I. are given the inividuals from no. 1

TABLE I.

THE FŒTUSES GROUPED ACCORDING TO LENGTH. The left-hand column gives the group number in lots of 50. The lowest space gives the totals for the individuals of given lengths.

	0–10		10–20		20–30		30–40		40–50		50–60		60–70		70–80		80–90		90–		
Group.	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	Total.
1.....			1	2	3	2	4	2	1	2	1	3	8	7	4	4	2	4			50
2.....	1		4	1	5	2	5	1	3	3	2	1	1	5	5	7	3	1			50
3.....	2		3	2	2	5		2	1	4	4	3	4	4	6	5	2		1		50
4.....			1		2	2	4	2	4	5	4	1	2	4	7	8	3		1		50
5.....			2	1	5	4	6	1	6	2	2	3	6	3	3	3	2		1		50
6.....	1		1	3	1	2	5	3	2	3	2	3	7	2	6	4	3	2			50
7.....			4	3	5	2		3	4	3	6	4	5	1	6	3	1				50
8.....			5	3	5	3	3	3	5	4	2	4		1	6	2	2	2			50
9.....				4	4	2	7		3	4	2	7	4	3	3	5	1	1			50
10.....	1	2	6	1	3	2	4	5	2	1	6	1	5	2	1	3	2	3			50
11.....	1		4	2	4	5	4	2	4	7	5	4	2	3	1	1			1		50
12.....			2	1	4	6	5	7	7	1	5	1	3	3	3	2					50
13.....			3	2	1	1	4	4	2	2	6	2	7	5	3	5	1	2			50
14.....				2	3	9	5	3	4	5	2	1	4	4	2	1	4			1	50
15.....		1	1	1	5	2	5	3	3	1	4	2	6	3	5	2	3	3			50
16.....			3	3	4	9	6	9	3	2	2	1	3		1		2	2			50
17.....				2	5	3	2	4	6	2	3	4	5	1	2	6	1	4			50
18.....	1	1	7	4	6	6	4	1	3	4	2	2	2	2	1	1	2	1			50
19.....	2		6	3	5	4	6	1	2	3	3	1	1	5	1	1	3	3			50
20.....	2		3	1	5	4	5	5	4	3	3	4	5	2	2	2					50
Total..	11	4	56	41	77	75	84	61	69	61	66	52	80	60	68	65	37	28	4	1	1000

to 50; next, from 50 to 100; 100 to 150 and so on. In the column to the extreme right is given the total for each space as a check on the number of individuals, the total for all spaces being 1,000. The group totals for different lengths, given in the lowest space, show the males without exception to be more numerous than the females, although in the 20–30 group the sexes approach equality. In all, there are 552 males and 448 females, giving a sex ratio of 123.21. If the sex ratio for each of these groups is determined we get the following values (Table II.):

It is obvious that in the 0–10 group and the 90–100 group the number of individuals, 15 and 5 respectively, is too small to be considered separately, especially since the sex ratio in each case is so extremely high. In the 10–20 group the sex ratio of 136.5 is well above the sex ratio of 123.21 for all individuals, while in the 20–30 group it has fallen to 102.6. Then in the next four groups from 30 cm. to 70 cm. the average sex ratio is 127.75.

Therefore, the results in the 20–30 group are very peculiar and can be interpreted only as due to chance, even though the number within that group is 152 individuals. It would be impossible for the males to reach again such a preponderance as 127 to 100 females after such a differential elimination of the sexes as would appear to be indicated in the 20–30 group, if there were such a high mortality of males in that group.

TABLE II.

SHOWING SEX RATIOS ACCORDING TO LENGTH.

Length.	Sex Ratio.	Number of Individuals.
0– 10	275.0	15
10– 20	136.5	97
20– 30	102.6	152
30– 40	137.7	145
40– 50	113.1	130
50– 60	126.9	118
60– 70	133.3	140
70– 80	104.6	133
80– 90	132.1	65
90–100	400.0	5

If the sex ratios are computed for individuals up to a certain length on the one hand and then for all over that length, a comparison can be made of the ratios for *relatively* younger and older fœtuses. In this way if there is any difference in the viability of the sexes we should expect to find that in the younger fœtuses the sex ratio would be high and that there might perhaps be a critical age, as indicated by length, in which there would be a greater mortality for one sex than for the other. We should also expect to find that after this critical stage in development is past the sex ratio in the remaining groups would not vary so greatly from the total of 123 as it did up to that group.

Table III. gives such a grouping with the number of individuals and the sex ratio for each group. The advantage of greater numbers within the group is also gained in this way. The sex ratios in this table were computed from the complete tabular list.

Thus, it is noted that for 112 individuals up to 20 cm. the sex ratio is 148.88 and that for the remaining 888 individuals from

TABLE III.

SHOWING A COMPARISON OF THE SEX RATIOS OF RELATIVELY YOUNGER AND OLDER FŒTUSES.

Length.	Individuals.	Sex Ratio.	Length.	Individuals.	Sex Ratio.
0– 20....	112	148.88	20–100	888	120.34
0– 30.....	264	120.00	30–100	736	124.39
0– 40.....	409	125.96	40–100	591	121.34
0– 50.....	539	123.14	50–100	461	123.78
0– 60.....	657	123.46	60–100	343	122.72
0– 70.....	797	125.14	70–100	203	115.95
0– 80.....	930	121.95	80–100	70	141.37
0– 90.....	995	122.50	90–100	5	400.00
0–100.....	1,000	123.21			

20–100 cm. the sex ratio is 120.34. The objection may be raised, and quite rightly, that 112 is too small a number on which to base definitely the sex ratio for individuals from 0–20 cm., especially since, when the next group of individuals from 20–30 cm. is included, the sex ratio from 0–30 cm. becomes lowered to 120.0. However, it should be pointed out that in the 20–30 cm. group, there is a relatively low sex ratio of 102 when compared with the total of 123, and that this is probably due to chance. When this is taken with the sex ratio of the 0–20 cm. group with a sex ratio of 148, it of course lowers it considerably and the reverse is true for the individuals above that length. Thus, the groups from 30–100 cm., being relieved of the burden of the 20–30 cm. group, have a combined sex ratio of 124.39. This no doubt partly explains such discrepancies as appear in passing from the 0–20 cm. to the 20–30 cm. groups, and in the 20–100 cm. and the 30–100 cm. groups. If the 20–30 cm. group had been such that it could have been considered practically normal, the wide and suddenly marked differences here shown probably would not exist.

However, it might be suggested that, although the sudden drop in the number of males in the 20–30 cm. group may represent a chance occurrence, it might be that this is the critical period in embryonic development in which there is a greater viability in the female fœtuses and that the normal sex ratio for this group might well be below the average of 123.

In the 0–40 cm. group the sex ratio of 125.96 shows a greater sex ratio than in the 40–100 cm. group with a sex ratio of 121.34, and here again, as in all succeeding cases, the 20–30 cm. group tends to lower the sex ratio for the relatively younger individuals. In the 0–50 cm. and the 50–100 cm. groups there is an approximate equality between the sexes, being 123.14 in the former and 123.78 in the latter case. The sex ratio of the groups, at which so close an approximation is reached, is also practically the same as the sex ratio of 123.21 for the total number of individuals. The same is practically true of the 0–60 and the 60–100 groups with a sex ratio of 123.46 and 122.72 respectively. In the 0–70 cm. group with 797 individuals, there is a slight rise in the sex ratio (125.14) and for the 70–100 cm. group with 203 individuals the ratio is 115.95. In the group from 0–80 cm. with 930 individuals the sex ratio of 121.95 of course approaches the average and in the 80–100 cm. group with only 70 individuals there is a sex ratio of 141.37, which can, in so small a number, likewise be considered as due to chance. From 0–90 cm. with 995 individuals the sex ratio is 122.5 and the remaining five have a sex ratio of 400.

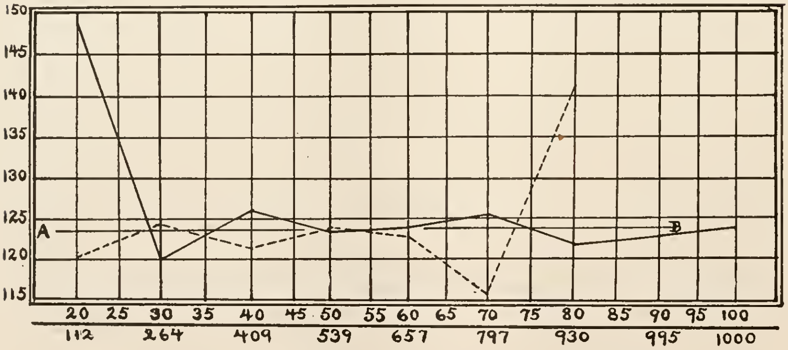

Fig. 1. Representing graphically the sex ratios for relatively younger and older fœtuses. The values for the sex ratios are indicated on the ordinate and the lengths on the abscissa. The number of individuals in each case is given below the lengths. Those below the dividing line (relative to length) are indicated by the continuous line, those below that length by the dotted line.

This is represented graphically in the following figure (Fig. 1) in which the fœtuses below the dividing line (relative to length) are shown by the continuous line.

The values for the sex ratio are indicated on the ordinate and the lengths of the individuals on the abscissa. The number of individuals dealt with in each case is given below the length. From 30 cm. and upward the highest point is 125.96 in the 0–40 cm. group, and the lowest is 120 in the 0–30 cm. group. The average sex ratio from 30–100 cm. is 123.27 as indicated by the line AB and the average deviation in this group is therefore 2.88.

If the line for all individuals over certain lengths is plotted in conjunction with those below the same lengths, the relative sex ratios for the groups compared can be seen. This is done in Fig. 1, the values being taken from Table III. From this figure it will be seen that on the average the dotted line runs below the heavy line, the former representing the individuals above the lengths indicated and the latter being those below the same lengths. The exceptions to this are in the 20–30 cm. group and the 80–100 cm. group with a sex ratio of 141, which includes only 70 individuals.

Therefore it would seem that in the younger stages there may be a slight difference in the viability of the two sexes, the male being somewhat more susceptible to intrauterine disturbances. However, this difference does not seem to be constant enough to be of real significance and one could not, from the data given here, conclude with certainty that in the fœtal development in cattle there is a greater mortality in the males during any particular developmental stage. A larger collection of individuals within each group would no doubt clarify the situation, although with all groups considered together, the sex ratio of 123 is probably very near to the true value.

Discussion.

A. *Reasons for Discrepancies Between the Primary and Secondary Sex Ratios in Cattle*

1. *A Possible Differential Viability.*—The only data for the secondary sex ratio with which we can compare the ratios during fœtal development are those of Wilckens ('87) who places the secondary sex ratio at 107.3; Pearl and Parshley ('13) who find it to be 113.3 in a population of 480, and Pearl ('17) who finds it to be 100.12 in a population of 1,313 individuals.

If we can compare the data obtained during fœtal development with that given above as the ratio at birth, it will be noticed that the former on the whole, with a sex ratio of 123.21, is well above the secondary ratio. Since in all of these cases the data include various breeds, it is fair to compare them in this respect. It would thus appear that there is a greater mortality among the male fœtuses, for only in this way would the sex ratio at birth be less than that during fœtal development. The figures as they stand in the present article, however, do not show sufficiently well-marked evidence to support the supposition that there is a greater mortality among the males at any particular stage of development.

2. *Possible Variations in Different Populations or Breeds.*—During the summer of 1919 correspondence was carried on with a large number of breeders to obtain data for comparison of the sex ratio at birth with the ratio during fœtal development, and also to determine the influence, if any, of the breed on the sex ratio. The Short Horn was taken as the beef type and the Holstein-Friesian as the dairy type of cattle. Only registered cattle were used and the data included the sire's name and number, the year, and the number of male and female calves by that sire. The total results of this investigation are given in Table IV. Data were also obtained in reference to twins of the same sex and of opposite sexes.

TABLE IV.

SHOWING THE SEX RATIOS FOR SHORT HORNS AND HOLSTEIN-FRIESIANS.

	Short Horns.			Holstein-Friesians.		
	Males.	Females.	Ratio.	Males.	Females.	Ratio.
Single births	862	881		958	978	
Pairs of twins of same sex	2	2		15	17	
Pairs of twins, opposite sex	8			18		
Single births and twins of opposite sex	870	889	97.86	976	996	97.99
Total, including all twins	874	893	97.87	1,006	1,030	97.66
Grand total, both breeds	Males 1880; females 1923. Sex ratio, 97.76					

From this table it will be seen that the total number of single births and dizygotic twins in the Short Horns included 870 males and 889 females with a sex ratio of 97.86. The corresponding group in the Holstein-Friesians contains 976 males and 996 females with a sex ratio of 97.99.

In the Short Horns the total including the twins of the same sex, a very few of which *might* have been monozygotic, there are 874 males and 893 females, the ratio being 97.87. The corresponding group of Holstein-Friesians contains 1,006 males and 1,030 females with a sex ratio of 97.66.

The total in both breeds was 1,880 males and 1,923 females, making a total population of 3,803 with a sex ratio of 97.76.

Thus it will be seen that there is no marked variation in the sex ratios of the two breeds, the two in fact approximating each other very closely.

It is possible that in a mixed population there might be a change in the sex ratio as has been indicated by various investigators in data on hybridization. Guyer ('09) found a great excess of males among hybrid pigeons and suggested that "more or less default in the metabolic processes because of the incompatibilities which must necessarily exist between two germplasms so dissimilar" would lead to a production of more males.

M. Pearl and R. Pearl ('08), in comparing pure with cross matings for man in Buénos Ayres, showed a "significantly greater proportionate production of males in the offspring from matings involving different racial stocks than in the offspring from matings in which both parents belong to the same racial stock."

Unusual sex ratios might result in hybridization of breeds of cattle that were unlike in respect to the size of the fœtus. An extreme case illustrative of this would be the bison-cattle crosses. Babcock and Clausen point out that practically all of the offspring of this cross are females due to the increased size of the hybrid fœtus and the consequent abortion or death of the male fœtuses. Thus, sex eliminating factors, as in this case, would change the secondary sex ratio.

Wilckens found that in the "Niederungsrassen" the sex ratio was 114 in 3,009 individuals as compared with an average ratio of 107.3.

3. *Errors in Sampling.*—It is also probable that errors might be made in sampling in the secondary and tertiary sex ratios. This might be done quite unconsciously by farmers, due to failure to record the sex promptly at birth. Some farmers might be prejudiced in favor of one sex and if the calves of the other sex should die before registration they probably would not be entered.

From this viewpoint one might compare the discrepancies in data on the sex ratios in cattle as given by various investigators, ranging from 97.76 (in Table IV. above) to 107.2 in the data of Wilckens and 113.3 as given by Pearl and Parshley.

Numbers Involved.—The question might be raised as to whether the number of fœtuses, 1,000, would represent a small enough number to make a material difference in the sex ratio as compared with a much larger number. Considering the sex ratio in the larger groups of fœtuses when grouped according to length it would appear that the sex ratio for the total collection, 123.21, is very near to the true one during fœtal development in cattle.

B. *Association of Sexes in Fœtal Twins.*

The data indicates that the sex ratio of 134 for dizygotic, fœtal twins, as found by Dr. F. R. Lillie, is not far from the normal of the population and therefore the association of sexes in twins is presumably a random sampling.

Conclusions.

1. The sex ratio during fœtal development in cattle is 123.21

2. This sex ratio of 123.21 during fœtal development compared with the much lower sex ratios at birth indicates a greater mortality of males during intrauterine development.

3. When compared with the sex ratio of 123.21 the sex ratio of 134 in F. R. Lillie's collection of twins in fœtal cattle does not indicate any interference with the chance assortment of sexes in dizygotic twinning in cattle.

4. The data do not indicate that there is any particular stage in development during which there is a more marked mortality among the males than in any other stage.

5. There is no indication that the breed causes any variation in the sex ratio.

6. Discrepancies between the primary and secondary sex ratios in cattle, excepting errors of sampling, are due to sex eliminating factors rather than factors that effect the primary sex ratio.

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A CORRECTION CONCERNING THE LIFE ZONES OF CANADA.

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A great step forward in the study of North American biota was taken when Merriam ('94) divided the continent into life zones and faunal areas on the basis of temperature. His divisions were natural ones and have been widely adopted, though they have been objected to by some botanists who have, however, given us nothing better, or as good. Subsequently several faunal maps of North America have appeared, founded mainly on that of Merriam ('98), and in some of these, notably that of Seton ('09), faunal areas not previously recognized are marked.

All the faunal maps of North America that I have seen are inaccurate in regard to one particular of which I happen to have special knowledge—the border-line between the Transition and Canadian Zones in Ontario. In Merriam's and Seton's maps the top of the Bruce Peninsula is indicated as being in the Canadian Zone, and the line between the Canadian and Transition is a little too low at the point where it touches Georgian Bay. In the American Ornithological Union map (1910) not only the whole of the Bruce Peninsula but a good deal of Central Ontario is indicated as Canadian.

The data which I here make use of in showing the true faunal position of the Bruce Peninsula was obtained while engaged in biological work on the peninsula in 1905, '07, '08, '09, '10, '11, '12 and '15, and that concerning the position of the boundary between the two zones in the vicinity of Georgian Bay was obtained on a motor-boat trip round the bay in 1912, when I camped at various points on the shore, and at Lake of Bays, Muskoka, in 1916. Work done in the vicinity of Ottawa in 1917 confirms the position of the line between the two zones as given by Merriam and Seton.

The Transition Zone, as its name implies, is a region of intergradation between the Canadian and Upper Austral Zones, and is not characterized by a number of species peculiar to itself but by a fauna and flora made up of southern and northern elements. The Canadian Zone, on the other hand, is characterized by many species. It is forested with coniferous trees, in the east mainly with white spruce (*Picea canadensis*), red spruce (*Picea rubra*) and jack pine (*Pinus banksiana*).

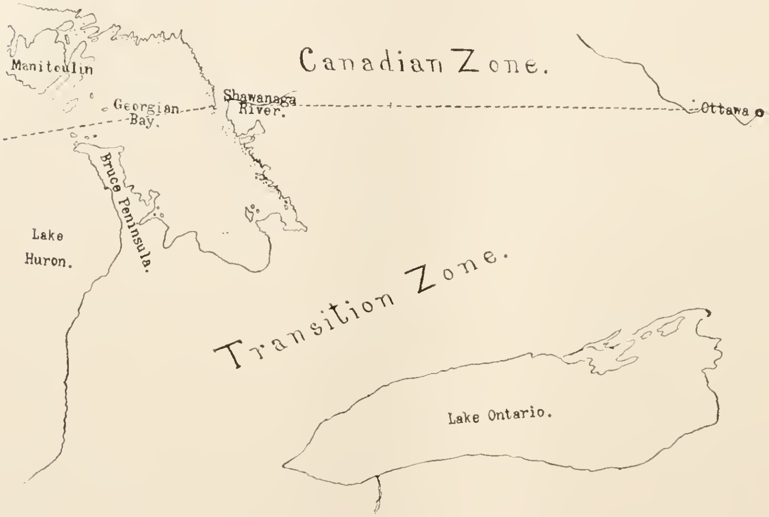
Among the characteristic mammals are the Canada lynx, marten, Canada porcupine, varying hare, northern red squirrel, star-nosed mole, northern flying-squirrel and northern jumping-mouse, and among birds the spruce grouse, arctic three-toed woodpecker, olive-sided flycatcher, Canada jay, American crossbill, white-throated sparrow, slate-colored junco, Blackburnian warbler, Tennessee warbler, magnolia warbler, bay-breasted warbler, myrtle warbler, olive-backed thrush and hermit thrush.

If any region has a flora and fauna consisting of many Austral species and very few Canadian, it is clearly in the Transition Zone. On the Bruce Peninsula, and on Cove, Bear's Rump, and Flower-pot Islands off the top of the peninsula, white spruce occurs but is scarce, red spruce is absent, and the predominating forest is maple-beech. The following Austral plants, which have an extensive range to the southwest, grow on the peninsula—*Gentiana procera*, *Linum medium*, *Satureja glabra*, *Solidago riddellii* and *Cacalia tuberosa*, and also the following species which have an extensive range to the south—*Eleocharis acuminata*, *E. rostellata*, *Scleria verticillata*, *Scirpus lineatus*, *Trillium grandiflorum*, *Viola rostrata*, *Rosa carolina* and *Uvularia perfoliata*. No typical Canadian plants, with the exception of *Carex scirpoidea*, occur on the peninsula, as *Linnæa borealis*, *Cornus canadensis*, *Clintonia borealis*, *Maianthemum canadense* and *Streptopus roseus* which are common, and which are sometimes considered Canadian species, extend far south into the Transition. A fuller discussion of the flora of the peninsula will be found in two of my papers (Klugh, '06, '12).

Among mammals it is the southern wild cat and not the northern Canada lynx that occurs on the Bruce Peninsula, and the Austral form of the red squirrel, *Sciurus hudsonicus loquax*, and not the northern *S. hudsonicus*.

The following Austral birds breed on the peninsula—red-headed woodpecker, Baltimore oriole, towhee, indigo bunting, migrant shrike, brown thrasher, catbird, prairie warbler (see Saunders '06, Klugh '09, '10), wood thrush and bluebird, while of the Canadian birds mentioned above only the white-throated sparrow, Blackburnian warbler, myrtle warbler and hermit thrush breed, the two latter being rare as summer residents.

The above data shows conclusively that the Bruce Peninsula is in the Transition Zone.



It is of course obviously impossible to adequately represent the boundaries between life-zones and faunal areas by a sharp line, since the change from one zone or area to another is gradual and not abrupt. But our lines should be drawn through the region where a comparatively few miles north or south shows an appreciable change in the biota. In the case of the line between the Canadian and Transition Zones in Ontario this is certainly not true if the line is drawn through central Ontario, nor is it true if it touches Georgian Bay low down. It is not until we reach the mouth of the Shawanaga River that we have a clearly Canadian fauna and flora to the north and a Transition biota to the south. That the line should be drawn as indicated on the accompanying map is shown by the data given by Fleming ('01) and Wright ('20) as well as by my data.

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THE PARTHENOGENETIC EFFECT OF ECHINODERM EGG-SECRETIONS ON THE EGGS OF NEREIS LIMBATA.

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(From the Marine Biological Laboratory at Woods Hole and the Zoölogical Laboratory of Amherst College.)

Various methods for inducing development in the eggs of *Nereis limbata* have been tried, but, until now, only two have been successful. Fischer ('03), working in Loeb's laboratory, found that *Nereis* eggs would divide and develop to the trochophore stage if treated for half an hour with sea-water which had been made hypertonic by the addition of NaCl, KCl or sugar. Just ('15) obtained the same results by putting the eggs into sea-water warmed to 35° or 36° C. Since all other methods tried had been unsuccessful, *Nereis* acquired the reputation of being refractory to parthenogenetic methods.

Because of this reputation, I decided to see whether or not development could be induced by means of the substance secreted into sea-water by echinoderm eggs. The experiments, as shown in the table, were successful. In performing them, fresh "dry" *Nereis* eggs were allowed to stand ten minutes or more in the secretion and then transferred to sea-water, where a large proportion would exude jelly, form fertilization membranes, and become mature. Some of these, continuing development, would divide, form normal trochophores, and, finally, small worms, in every external appearance like those resulting from sperm fertilization. These have been kept until eighteen days old. It takes these parthenogenetic eggs about twice as long to reach a given stage as it does the sperm controls, a fact which serves as a nice check against accidental contamination with sperm.

Not only is development brought about by egg secretions, but the same effect may be obtained by treating *Nereis* eggs with the lipolysin obtained from the secretions (Woodward, '18).

TABLE I.

	40 sec.		1 min.		2 min.		3 min.		5 min.		7.5 min.		10 min.		20 min.		30 min.		45 min.		50 min.	
	Cleavage.	Swimmers.	Cleavage.	Swimmers.	Cleavage.	Swimmers.	Cleavage.	Swimmers.	Cleavage	Swimmers.	Cleavage.	Swimmers.	Cleavage.	Swimmers.	Cleavage.	Swimmers.	Cleavage.	Swimmers.	Cleavage.	Swimmers.	Cleavage.	Swimmers.
Asterias egg secretion													70%	1%	90%	3%	60%	Few	30%	1%		
Asterias lipolysin 1%					0	0			0	0			30%		40%				81%			
Asterias lipolysin 2%					Few	Few			Many	0			Few	Few	33%	1%			Many			
Arbacia egg secretion			0				20%		10%	1%			30%		30%		10%				1%	
Arbacia lipolysin 1%			1%		5%	1%	10%	Many	8%	Few			1%	.5%	1%	Few	2%	1%			12%	
			0		5%		2%		5%				18%		2%		4%				19%	
Arbacia lipolysin 2%			1%			3%	50%	Few		25%			30%	Few	50%	10%	60%			5%		5%
						Few		Few	Many		Many		Many									
	50%	0	50%	Few	40%	Many	35%	Few	70%	Few	60%	Few	50%	Few								
Echinarachnius egg secretion.													50% memb.		10% memb.		10% memb.		16% memb.			
Echinarachnius lipolysin 1%[1].				50%		1%		1%	3%													

The numbers in a horizontal row refer to experiments with a single batch of eggs.

[1] Many of these larvæ were still living eighteen days later.

Since a solution of lipolysin in sea-water may be made much more concentrated than the original secretion, it is not necessary to expose the eggs so long to its action. In fact, one of the most sensational results was the development to the trochophore stage of 50 per cent. of the eggs exposed for one minute to a solution of *Echinarachnius* lipolysin. Most of those eggs continued to develop, and formed segmented worms. The solution used, so-called 1 per cent., was made by dissolving .1 c.c. powdered lipolysin in 10 c.c. filtered sea-water.

In a third series of experiments, dilute *Arbacia* egg-secretion was passed through a Berkfeldt filter. In this process, the agglutinin remains on the filter and the lipolysin, with other substances, passes through. *Nereis* eggs which were left in this filtrate eighteen hours underwent maturation and divided regularly into eight or more cells. They were then transferred to sea-water, in which they continued to develop into normal larvæ.[1]

It was suggested by Dr. F. R. Lillie that the effect of egg-secretion was simply that of a foreign protein, and might be imitated by using cœlomic fluid. Following this suggestion, I subjected *Nereis* eggs to the cœlomic fluid from *Asterias*, treating them with various dilutions and for varying periods of time. While some eggs formed polar bodies and secreted jelly, only a few started to divide, and that very irregularly. I did not succeed in finding any method by means of which the serum would produce normal division or swimming larvæ.

These experiments with *Nereis* eggs continue a series begun several years ago by O. C. Glaser ('14) who found that *Asterias* eggs could be stimulated to divide by letting them stand in *Arbacia* egg-secretion. The following year I performed the reciprocal experiment, which helped to show that the parthenogenetic agent in egg-secretion is not specific. Later, I found that *Asterias* lipolysin produced as many larvæ in *Arbacia* eggs as did *Arbacia* lipolysin (Woodward, '18). The present work shows that the parthenogenetic agent in echinoderm egg-secretions is not even limited in its efficacy to eggs of the same phylum, but that it is effective in bringing about development in some annelid eggs, even though the latter are resistant to the usual agents.

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[1] This experiment was first performed by Miss M. M. Sampson and repeated by myself.

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STUDIES ON BIOLUMINESCENCE.

XIII. LUMINESCENCE IN THE CŒLENTERATES.

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INTRODUCTION.

During a recent trip to the Pacific coast, the opportunity presented itself of studying light production in a number of cœlenterates and of making observations on other luminous forms. These studies were carried out at the Puget Sound Marine Station, Friday Harbor, Washington, and the author expresses his keen appreciation of the facilities afforded him there by Professor T. C. Frye, director of the laboratory.

We are indebted to Panceri (1) for most of our knowledge of luminescence among the cœlenterates. The present paper supplements his work and gives the facts in the case of certain forms found in Puget Sound.

EXPERIMENTS ON HYDROMEDUSÆ.

Seven species of hydromedusan jelly-fish were obtained at Friday Harbor, namely, *Æquorea forskalea*, *Mitrocoma cellularia*. *Phialidium gregarium*, *Stomatoca atra*, *Sarsia rosaria*, *Melicerta* sp. ? and an unidentified form. One scyphomedusan, *Cyanæa* sp. ?, is also occasionally to be collected. Of these only the first four produce light and only the first three are markedly luminous. The first five were very common forms, especially *Æquorea*, *Mitrocoma* and *Phialidium*, obtained in the morning from the laboratory float where they live in water of rather low temperature. Their appearance is somewhat capricious, however, and on several days only one or two were seen.

Æquorea and *Mitrocoma*, 1½ to 3 inches in diameter, and *Phialidium*, about 1 in. in diameter, produced light of a bright bluish-green color (but not so blue as the ostracod, *Cypridina*) from spots along the edge of the umbrella at the base of the tentacles. No other regions of *Æquorea* or *Mitrocoma* phosphoresce, but at times faint light was to be observed coming from masses (gonads) along the four radial canals of *Phialidium*. *Melicerta* is weakly luminous about the manubrium and then only on rather vigorous rubbing between the fingers.

Examined with the microscope in the daytime, the margin of the umbrella of *Æquorea* discloses oval masses of yellow tissue corresponding in position with the luminous areas at night. In *Mitrocoma*, the yellow masses are much closer together forming an almost continuous line in some places. Since the luminous spots observed at night are also very close together in this form, it seems as if the yellow tissue must be luminous tissue. In *Cypridina* and the worm, *Tomopteris*, there are also very clearly visible yellow cells in the luminous gland but this is not true of the luminous organ of all forms. These yellow regions of the medusæ do not stain with neutral red, intravitam, a fact true also for the yellow cells of *Cypridina*. In *Phialidium* or *Stomatoca* yellow cells cannot be made out but this is possibly because of their small size.

Examined at night under the microscope, the luminous spots present a beautiful appearance. Under conditions which cause a cytolysis of the cell, such as addition of fresh water or saponin, one can clearly see that the light comes from granules which are rather large and have a definite boundary,—light discs. They are not mere points of light. They vary in size and will luminesce for some time, then flash out very brightly and the light intensity slowly fade. Sometimes there is the sudden appearance of a light disk and then gradual fading of the luminescence. In the luminous extract of *Cavernularia* (2) I have described a similar phenomenon, where, upon addition of fresh water, the light intensity suddenly increases, due to the flashing out of photogenic granules. Under the microscope the appearance is that of the starry sky.

By addition of saponin to the luminous tissue, we obtain a very bright light and this is the best method of exciting luminescence for examination with the spectroscope. This discloses a band of light extending from about $\lambda = .46\,\mu$ to $\lambda = .60\,\mu$. As far as I was able to make out the limits are the same for both *Æquorea* and *Mitrocoma*, perhaps a somewhat narrower band for the latter.

The light of these jelly fish only appears on stimulation or on dissolution of the cell. It appears on handling or electrical stimulation, or when the jelly fish is carried by the current against some objects in the water. On merely touching a jelly fish one cannot observe that any luminous secretion is definitely thrown into the water as in the case of *Cypridina*, but on very gentle stroking of the edge of the umbrella a mass of luminous material comes off which adheres to the fingers, or on tossing an animal on the surface of the water, abundant luminous material is liberated which causes the sea water to luminesce. It appears that the luminous material comes off in the slime so commonly secreted by these organisms. A similar behavior is exhibited by the Pennatulid, *Cavernularia*. It would seem that this is to be interpreted as an extracellular luminescence, although not so marked a one, certainly, as that of *Cypridina*. There remains, however, the possibility that we have here cells very easily ruptured, with discharge of their contents into sea water.

The luminous material of *Æquorea*, *Mitrocoma* or *Phialidium* can be dried over $CaCl_2$ and will give a bright light when again moistened.

A strip of the margin of the umbrella of *Æquorea* or *Mitrocoma* is easily cut off with scissors, giving a mass of tissue containing as little non-luminous material as it is possible to obtain. If this is squeezed through four layers of cheesecloth, there is obtained a luminescent extract which glows for some hours. In one case the light was still visible after nine hours. This extract behaves just as a similar one prepared from *Cavernularia* (2). When the luminescence disappears on standing, the addition of fresh water, gentle heating or cytolytic agents such as saponin, sodium glycocholate, chloroform, ether, or NaCl crystals again calls forth the luminescence. Tannic acid, strychnin, or phloridzin do not cause the light to reappear. Once the light has been caused to disappear by addition of saponin or Na glycocholate powder, the further addition of fresh water will cause no more light to appear.

Isotonic cane sugar solution does not call forth the production of light. The phenomenon is unquestionably one of cytolysis, by diminution of osmotic pressure, by heat, or by addition of specific substances. In the extract there are probably intact photogenic cells which dissolve with production of light. In addition I believe the solution of photogenic granules is also accompanied with the emission of light, because one can very easily see, under the microscope at night, the sudden appearance of a disc of light, too small to be the illumination of a cell, but capable of interpretation as the light from a single granule within the cell.

Extracts of *Æquorea* which should contain luciferase give no light with extracts of *Æquorea* which should contain luciferin. The same is true for *Mitrocoma* and for crosses of luciferin and luciferase of *Cypridina* with these two medusæ. Every attempt to demonstrate these substances has given negative results. The reasons for this are discussed in another paper, to appear shortly.

It is reported that many luminous forms produce no light in the daytime, the power only appearing with the approach of dusk or if the animals are kept in the dark for some time. This is true of some forms but not of these medusæ. The four kinds of luminous medusæ, *Æquorea*, *Mitrocoma*, *Phialidium* and *Stomatoca*, were collected in bright sunlight and brought by an assistant to the dark room where I had been adapting my eyes to the dark for one half hour. All four forms luminesced immediately on stimulation and just as brightly as at night. The ctenophore, *Bolina*, did not luminesce even after ten minutes, when brought into the dark from strong sunlight, but did luminesce after thirty minutes. There is no doubt that *Bolina*, a further discussion of which follows, is affected by sunlight but these four medusæ are certainly not. *Noctiluca* appears to have its luminescence inhibited by strong sunlight also.

Experiments on Ctenophores.

At Friday Harbor, three species of ctenophores occur, *Bolina* sp. ?, *Pleurobrachia* sp. ?, and *Beroë* sp. ?, but only the first was common during my stay. *Bolina* luminesces readily at night. *Pleurobrachia* did not luminesce even on crushing and *Beroë* only gives a diffuse flash of light on vigorous agitation.

The light of *Bolina* comes from cells along the swimming plates. According to Dahlgren (3), the luminous cells form a layer over the testis and ovary, along the water vascular canals. In the living animal I was unable to make out any yellow cells in this region, comparable to the yellow masses of *Æquorea* or *Mitrocoma*.

The light is of the same bluish-green color as the medusæ, but too faint and evanescent for a study of its spectrum.

Bolina is an exceedingly fragile ctenophore and contains much water and relatively little luminous material. The animals also appear to be easily fatigued and lose somewhat their power to luminesce on frequent agitation. Portions of the swimming plate tissue placed on a glass slide, as much of the water drained away as possible, and dried over $CaCl_2$ in the dark do not give light on again moistening with water. This is probably to be explained by the small amount of photogenic material present.

If Bolinas are pressed through four layers of cheesecloth there is obtained a luminescent solution which rather readily loses its power of luminescence. It again gives light on vigorous agitation or addition of cytolytic substances. It behaves as the extracts of medusæ and pennatulids. If fresh water is added, we have the appearance of dots of light just as in these extracts. The existence of luciferin and luciferase also cannot be demonstrated and extracts of *Bolina* give no light with *Cypridina* luciferin nor do heated extracts of *Bolina* give light with *Cypridina* luciferase.

Since the observations of Allman (4) it has been known that ctenophores would not produce light in the daytime. Peters (5) made quite a study of this in *Mnemiopsis* and found that mechanical stimulation accelerates the appearance of luminescence in darkness after previous exposure to light. The inhibition of luminescence is roughly inversely proportional to the intensity of the light which has previously illuminated them.

Bolina shows marked inhibition of luminescence as a result of previous illumination. Animals brought into a dark room from direct sunlight about 10 A.M. gave no light whatever on stimulation immediately or after five minutes, gave some light on stimulation after ten minutes, and a good luminescence after one half hour in the dark. The question at once arises as to the cause of this behavior. Are the cells incapable of being stimulated after exposure to sunlight or do they fail to manufacture photogenic substances as a result of exposure to sunlight? One alternative supposes the cell to contain photogenic material which for some reason cannot be oxidized; the other, that no photogenic material is formed in the sunlight and the disappearance of that which has been formed. Some evidence can be obtained for the latter view by breaking up the cells of ctenophores which have been previously exposed to daylight. If no luminescence is produced the effect of light must be to prevent the manufacture of photogenic material. If luminescence occurs on breaking up of the photogenic cells previously exposed to daylight, the inhibitive actions of light must be on the stimulation mechanism.

If Bolinas, which have been previously exposed to daylight, are crushed through four layers of cheescloth, no light whatever appears during the crushing or on adding fresh water to cytolyse the photogenic cells. Similar Bolinas, kept in the dark for one half hour, give a bright luminescence under the same treatment. If this extract of crushed Bolinas, which had been previously exposed to sunlight, is allowed to stand in the dark for one half hour and then fresh water added, no light will appear. Whole Bolinas after sun illumination will again luminesce if kept in the dark for one half hour. This shows that there is no preformed photogenic material in sunlight exposed Bolinas and that none can be formed in crushed material even in the dark. The sunlight must therefore act to prevent the formation of photogenic substance rather than to prevent its oxidation on stimulation. Why sunlight causes the disappearance of photogenic material already formed is a question awaiting solution.

Experiments on a Sea Pen, *Ptylosarcus*.

Ptylosarcus is dredged at Friday Harbor in fairly deep water. Some of the specimens may be two feet long. The colony consists of a stalk without fronds buried in the sand and a stem with fronds that bear polyps only along the outer edge. The polyps, but not the surface of the fronds, are luminous. The stalk is not luminous but the stem has two luminous areas running the length of it and one non-luminous area between these.

The colony is non-luminous except when stimulated. Then a yellow greenish light appears of a more yellow hue than *Æquorea*. If the polyps are gently rubbed, a luminous slime comes off and the secretion can be seen in the sea water. The polyps, ground in sea water with sand, give a luminescent secretion which becomes very brightly luminescent on addition of fresh water, saponin and other cytolytic agents. The extract behaves exactly as a similar one prepared from *Cavernularia* and already described (2). In every way the behavior of *Ptylosarcus* agrees with that of *Cavernularia*.

It was impossible to demonstrate the presence of luciferin or luciferase in *Ptylosarcus*. The following "crosses" were also made, using extracts, which, from mode of preparation, should have contained *Ptylosarcus* luciferin and luciferase.

Ptylosarcus luciferase × *Ptylosarcus* luciferin—negative.
Ptylosarcus luciferase × *Æquorea* luciferin—negative.[1]
Æquorea luciferase × *Ptylosarcus* luciferin—negative.
Cypridina luciferase × *Ptylosarcus* luciferin—negative.
Ptylosarcus luciferase × *Cypridina* luciferin—negative.

Ptylosarcus brought into a dark room from direct sunlight was observed to luminesce on immediate stimulation and as brightly as at night. There is no inhibitive influence of light in this form.

Experiments on a Sponge, *Grantia*.

The question of luminosity in sponges is in rather of an unsettled state. Some observers have reported luminescence but Dahlgren (3) was inclined to attribute the light of a sponge obtained at Naples to luminous worms and protozoa living in its canals.

At Friday Harbor there exists a sponge, *Grantia* sp.?, one to three inches long, common on logs, piles, etc., in the sea water. If rubbed, a yellowish luminescence may be observed which can be obtained from all parts of the organism. If the sponge is crushed the luminescence is quite bright. Every individual of this kind of sponge examined showed luminescence, whereas another sponge, *Esperella* sp.?, living on *Pecten* shells, was not luminous. A few isolated dots of light only appeared on rubbing.

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[1] Sometimes a faint light was observed whose significance is unknown.

Sponges kept in sunlight for one half hour gave as good a luminescence as those in the dark.

Whether this is a true luminescence or whether due to luminous organisms living on the sponge cannot be definitely stated. The sponge could not be stimulated to luminesce electrically (interrupted induced currents) under conditions when jelly-fish showed a good luminescence. Examined under the microscope, no hydroids, radiolaria, dinoflagellates or Noctilucæ could be observed, but many desmids, diatoms, worms and infusoria. These forms are not luminous, however.

When squeezed through cheesecloth a luminous extract was obtained, the light coming from points of light in the extract as in the case of *Cavernularia* or medusæ. Addition of fresh water or saponin causes a great increase in light just as in extracts of cœlenterates. No luciferin or luciferase could be demonstrated.

The fact that an extract of this sponge made by squeezing through cheesecloth remains luminescent for some time is significant. If the light came from small luminous forms living on the sponge, we should expect them to pass through the meshes of the cheesecloth unharmed and then light would appear in the extract only on stimulation by agitation or in some other way. It is also significant that no inhibitive effect of sunlight was to be observed. Sunlight is said to inhibit the luminescence of many small organisms, especially dinoflagellates, which might live on the sponge. In general characters, the extracts so closely resemble those obtained from cœlenterates that I am inclined to believe the light of this species of sponge is a true luminescence.

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THE EXPLOSION OF THE SPERMATOZOA OF THE CRAB *LOPHOPANOPEUS BELLUS* (STIMPSON) RATHBUN.

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(forty-six figures.)

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Introduction.

For a number of years the writer has been studying the male germ cells of the Decapoda with two purposes in mind: (1) to discover the means by which the mature, dormant spermatozoa of the Decapoda become activated, in order to shed light on the problem of fertilization in this order of Crustacea, and (2) to trace more clearly the process of spermatogenesis. The present paper on the explosion of the spermatozoa of the black-clawed crab, *Lophopanopeus bellus*, is a contribution involving the former of these problems.

Material and Methods.

The material for this study consisted of the living spermatozoa of *Lophopanopeus bellus*, common in certain localities around the Puget Sound Biological Station, Friday Harbor, Wash. The spermatozoa of this crab are very favorable for study in that they are not enclosed by the numerous spermatophores so common in other brachyura. As pointed out in another paper (Fasten, 1917), "in *Lophopanopeus bellus* it doesn't seem as if numerous spermatophores are developed. Here it appears that one large spermatophore is formed in which the spermatozoa are tightly packed." Since this is the condition all that was necessary to obtain a plentiful supply of living spermatozoa was to rupture the deferent ducts and the male gametes oozed out in tremendous numbers.

The living spermatozoa were studied in the same manner as described in my earlier paper on the spermatogenesis of the edible crab, *Cancer magister* Dana (Fasten, 1918). Numerous spermatozoa suspended in the crab's body fluid, or in sea water which is isotonic with the crab's body fluid, were placed on a slide and covered with a cover glass. These could then be studied with the high power oil-immersion lenses. By allowing various chemical solutions to diffuse under the cover glass all changes in the spermatozoa could be observed and outlined with the aid of the camera lucida.

The living spermatozoa were studied in the following solutions:

1. Crab's body fluid.
2. Sea water.
3. Sodium chloride ($NaCl$)—M/2 $NaCl$ and less.
4. Sodium nitrate ($NaNO_3$)—M/2 $NaNO_3$ and less.
5. Calcium chloride ($CaCl_2$)—3/8M $CaCl_2$ and less.
6. Potassium chloride (KCl)—M/2 KCl and less.
7. Potassium nitrate (KNO_3)—M/2 KNO_3 and less.
8. Potassium hydroxide (KOH)—very dilute solution.
9. Distilled water.
10. Cane sugar ($C_{12}H_{22}O_{11}$)—M/1 $C_{12}H_{22}O_{11}$.
11. Ovarian fluid.
12. Acidulated sea water. Various small amounts of acids were added to sea water, such as: glacial acetic, salicylic, saponin, sodium glycocholate, nitric, hydrochloric, oxalic, tannic, picric, and chromic acids.

Many of the spermatozoa in all stages of explosion were fixed on the slide with either osmic acid fumes, or Bouin's fluid, or Flemming's mixture, and then stained with Heidenhain's iron-hæmatoxylin and acid-fuchsin. Those fixed with osmic acid fumes gave beautiful results, so that the stained elements were perfect representations of the living structures. This can be clearly seen when one examines Figs. 3–7, which are from stained preparations fixed with osmic acid fumes, and compares them with figures 1 and 2 which are from living spermatozoa suspended in the body fluid of the crab.

Normal Appearance of Spermatozoa.

The living spermatozoa of *Lophopanopeus bellus* when studied in the cœlomic fluids of the crab are found to be small, greenish bodies, which appear like spheroids when seen from the top or bottom (Fig. 1), and like ellipsoids when viewed from the side (Fig. 2). In structure they seem to be similar to those of *Cancer magister*. Within the centre there is a clear central body (Figs. 1 and 2, *b*) and surrounding this are two vesicles; a uniform, darkly green secondary vesicle (Figs. 1 and 2, *v'*), and a clear, transparent primary vesicle (Figs. 1 and 2, *v*). Outside of these vesicles is a granular and vacuolated protoplasmic cup (Figs. 1 and 2, *h*) of a lighter greenish hue than the secondary vesicle. If the spermatozoa remain suspended in the crab's body fluids for some time their protoplasmic cups open up and liberate the radial arms (Figs. 3–7). It is thus seen that the protoplasmic cup of the spermatozoön consists of a nuclear cup (Fig. 3, *n*) and radiating radial arms (Fig. 3, *r*).

When the spermatozoa are fixed with osmic acid fumes and stained by the iron-hæmatoxylin and acid-fuchsin methods, then the nuclear cup, radial arms and the central body stain black (see Figs. 3–7), the second vesicle stains a dark amber, whereas the primary vesicle remains transparent.

Four types of spermatozoa are produced, depending on their number of rays. There is a three (Fig. 4), four (Fig. 5), five (Fig. 6), and a six (Fig. 7) rayed type. The four (Fig. 5) and five (Fig. 6) rayed types, however, are produced in largest numbers. These rays are not pseudopodia-like processes similar to those which Binford ('13) pictures for the spermatozoa of *Menippe mercenaria*. They are distinct arms similar to those found in the crayfish *Cambarus virilis* and *Cambarus immunis*, as pictured by the writer in a previous paper on the spermatogenesis of these forms (Fasten, 1914).

Effects of Chemical Agents on Spermatozoa.

1. *Sea Water*.—Sea water produces no change in the normal appearance of the spermatozoa. The protoplasmic cup, however, swells slightly and liberates the radial arms (Figs. 8 and 9).

2. *Sodium Chloride*.—An M/2 NaCl solution which is isotonic

with sea water produces no change in the normal appearance of the spermatozoa (Figs. 10 and 11). An M/4 NaCl solution brings about a slight shrinkage in the nuclear cup, otherwise there is no further change. In an M/6 NaCl solution the secondary vesicle is very slowly everted. First of all it squeezes out in the form of a small bubble (Fig. 12), until very gradually it assumes the appearance shown in Fig. 13. In an M/7 NaCl solution the eversion of the secondary vesicle is much faster. Figs. 14, 15 and 16 show successive stages in the eversion process. Most of the spermatozoa proceed to the stage shown in Fig. 16 and then cease. An M/8 NaCl solution brings about a complete and rapid explosion of all the spermatozoa. Figs. 17, 18 and 19 show respectively the beginning, middle and end of the process. In Fig. 19 the secondary and primary vesicles, as well as the central body are seen completely everted.

3. *Sodium Nitrate.*—An M/2 $NaNO_3$ solution which is isotonic with sea water brings about no appreciable change in the normal appearance of the spermatozoa. In an M/4 $NaNO_3$ solution the only change noticed in the spermatozoa is a slight swelling of the nuclear cup. An M/8 $NaNO_3$ solution causes a slow eversion of the secondary vesicle, producing figures similar to those shown in Figs. 15 and 16. In an M/16 $NaNO_3$ solution the eversion of the two vesicles occurs rapidly and with considerable force, so that all the spermatozoa soon take on the appearance shown in Fig. 19.

4. *Calcium Chloride.*—A 3/8M $CaCl_2$ solution is isotonic with sea water and this brings about no change in the normal spermatozoa. A 3/11M $CaCl_2$ solution brings forth a partial eversion of the secondary vesicle (Fig. 20). In a 3/16M $CaCl_2$ solution the spermatozoa explode completely. The vesicles are entirely everted and at the same time the nuclear cup shrinks considerably and becomes irregular. Figs. 21–24 show various stages in the explosion process. In the $CaCl_2$ solutions the detailed structure of the spermatozoa can be clearly distinguished.

5. *Potassium Chloride.*—In an M/2 KCl solution which is isotonic with sea water the spermatozoa remain normal. In M/4 and M/8 solutions of KCl the only perceptible change produced in the spermatozoa is a disappearance of the granules

and vacuoles in the nuclear cup making it become more homogeneously green. Also the secondary vesicle shrinks somewhat, thereby leaving the clear primary vesicle to show more prominently (Fig. 25). An M/16 KCl solution produces swelling and explosion of the spermatozoa (Fig. 26). In many instances the explosion is so violent that the nuclear cup ruptures completely.

6. *Potassium Nitrate.*—An M/2 KNO_3 solution which is isotonic with sea water does not produce any explosion. However, the protoplasmic cup swells and becomes more homogeneous in appearance. Also the primary and secondary vesicles become more distinctly marked off from each other (Fig. 27). An M/4 KNO_3 mixture has a similar effect. An M/8 KNO_3 solution brings about a swelling of the protoplasmic cup and a slow eversion of the second vesicle so that the spermatozoa resemble Fig. 28. In an M/16 KNO_3 solution the spermatozoa explode very rapidly and they come to look like Fig. 26.

7. *Potassium Hydroxide.*—Very dilute solutions of KOH bring forth a violent reaction in the spermatozoa. The protoplasmic cup swells, becomes homogeneous and at the same time pushes the vesicles upward (Figs. 29–31). The secondary vesicle undergoes a rotation and is pushed to one side. Finally the vesicles explode with great violence and the entire spermatozoön soon goes to pieces.

8. *Distilled Water.*—Distilled water produces a rapid eversion of the vesicles so that in a very short time the spermatozoa come to resemble Figs. 32 and 33.

9. *Cane Sugar.*—From the above experiments two conclusions might be inferred regarding the explosion of the spermatozoa, one is that it is due to lack of electrolytes, and the other is that the explosion is due to a reduction of the osmotic pressure produced by surrounding the spermatozoa with a hypotonic solution. In order to determine which factor we have to deal with, the spermatozoa were surrounded with an M/1 cane sugar solution which is approximately isotonic with sea water. If the factor involved were due to lack of electrolytes then, since the sugar solution contains no electrolytes, the spermatozoa ought to explode. But the M/1 cane sugar solution did not produce any

change in the normal appearance of the spermatozoa, thereby pointing to the second factor, namely, osmotic pressure, as the one which undoubtedly operates in bringing about the eversion of the vesicles.

10. *Ovarian Fluid.*—Since a reduction in osmotic pressure produces the explosion of the spermatozoa, the next question which naturally arises is whether the female gonads at the time of fertilization produce a hypotonic substance which, when coming in contact with the spermatozoa, causes them to explode, thereby bringing about fertilization of the ova. In order to test this out, the ovaries and oviducts were mashed up in sea water and the living spermatozoa were then surrounded by this mixture. In some cases (not all), a few of the spermatozoa exploded violently. The nuclear cup at first swelled and became homogeneous (Fig. 34). Then the vesicles were everted with considerable force and in many instances, the primary vesicle, or both the primary and secondary vesicles completely disintegrated, leaving stages like those shown in Figs. 35–39. Whether this was due to some agent produced by the female gonads or to some other agent cannot be definitely stated, for not all of the spermatozoa were affected in the same manner as those mentioned above. However, it is also significant that the ovaries used during the months of the year when the investigations were conducted (June and July), were past maturity. They were small and immature and this might account for the results obtained. Another significant fact to be taken into consideration is that in control experiments in which living spermatozoa from the same males as those used in the experiments with the ovarian fluids, were surrounded with sea water alone, none of the spermatozoa exploded. Now, the question arises, why should we get a violent explosion of even a few spermatozoa when ovarian contents are used and no explosion when the ovarian fluids are lacking? I am strongly of the opinion that the female gonads produce some substance which is responsible for the explosion. Also, it seems very probable that at the time of sexual maturity of the female this specific substance must be present in such quantities as to activate all of the living spermatozoa.

11. *Acidulated Sea Water.*—In all cases weak dilutions of the

acids were used. If the acid was a liquid, the dilution used was 1 part of the concentrated acid dissolved in 25 parts of sea water. A drop of this was then added to the edge of the cover glass under which the living spermatozoa were held suspended in sea water. If the acid used was crystalline in texture, then a few of the crystals were placed at the edge of the cover glass and allowed to dissolve slowly under it.

(*a*) *Glacial Acetic Acid.*—Causes the protoplasmic cups to lose their granular and vacuolated appearance. Usually two or three dark granules remain in the nuclear cup. The nuclear cups and radial arms swell and lose their color (Figs. 40 and 41). The spermatozoa in many instances are thrown together into aggregates (Fig. 42). After remaining exposed to the action of the acid for some time many of the spermatozoa explode (Fig. 43) and disintegrate completely.

(*b*) *Salicylic Acid.*—Reaction here is similar to that caused by glacial acetic acid.

(*c*) *Saponin.*—Causes considerable swelling (Fig. 44). Nuclear cup and radial arms become more homogeneous and much paler in color. They appear almost transparent. A few of the spermatozoa explode after being exposed for some time.

(*d*) *Sodium Glycocholate.*—Causes swelling similar to that produced by saponin or glacial acetic acid. During this swelling the vacuoles of the nuclear cup at first enlarge and then disappear, giving the nuclear cup a homogeneous appearance. Soon a violent explosion of vesicles takes place. Nuclear cup now loses its greenish color, becomes ragged and transparent with small dark spots. Very shortly the spermatozoa disintegrate.

(*e*) *Nitric Acid.*—This brings about a homogeneity of appearance in protoplasmic cup with considerable shrinkage (Fig. 45). The second vesicle in many cases is everted (Fig. 46).

(*f*) *Hydrochloric Acid.*—The reaction here is very similar to that caused by nitric acid.

(*g*) *Oxalic Acid.*—Reaction is similar to that produced by nitric acid.

(*h*) *Tannic Acid.*—Reaction is similar to that of nitric acid, with the exception that none of the vesicles are everted.

(*i*) *Picric Acid.*—The reaction produced in the spermatozoa is the same as that brought about by tannic acid. The spermatozoa are soon killed and stained a yellowish-green.

(*j*) *Chromic Acid.*—This produces a similar result to that obtained with either tannic or picric acids. Here the spermatozoa are fixed a yellowish-brown.

Discussion.

A careful examination of the data presented in this paper shows quite clearly that a lowering of the osmotic pressure in the medium which surrounds the spermatozoa is responsible for their explosion. In this respect the present research bears out what Koltzoff ('06) first suggested for the explosion of the spermatozoa of other decapods. Also, Binford ('13) in *Menippe mercenaria* and the present writer in *Cambarus virilis* (Fasten, '14), and *Cancer magister* (Fasten, '18), have found that osmotic pressure accounts for the explosion of the spermatozoa. In the light of all this accumulated evidence it seems quite certain that the stimulating agent which brings about the explosion in the spermatozoa of the Decapoda, is one which reduces the osmotic pressure in the medium that surrounds them.

Since this is the operating factor, the question which naturally suggests itself is where in the Decapoda is such a stimulating agent produced? The writer is strongly of the opinion that the mature gonads of the female decapod produce some chemical substance which, when it comes in contact with the spermatozoa, brings about their explosion. The experiments with the ovarian fluids seem to point to such a conclusion. The work of Koltzoff ('06) and Binford ('13) also suggests a similar conclusion.

Concerning the function of the explosion, it, undoubtedly, acts as the force or the motive power which drives the spermatozoön into the egg during the process of fertilization. What parts of the spermatozoön actually penetrate the ovum during fertilization is still a debated question. Koltzoff ('06) and Spitschakoff ('09) are in agreement that the nuclear cup (derived from the nucleus of the spermatid) is the only structure which enters the ovum. Binford ('13), on the other hand, claims that the everted vesicles (cytoplasmic structures) of the exploded spermatozoön are driven into the egg, whereas the nuclear cup remains on the outside where it soon disintegrates. In order to bring this mode of fertilization in harmony with the idea of the continuity of the chromosomes, Binford suggests that "the contents of the capsule (vesicles) may be derived from the nucleus of the spermatid and is probably oxychromatin which deposits basichromatin after it enters the egg and so gives rise to the chromosomes in the male pronucleus."

It is thus obvious that more research along this line is essential before any definite conclusions regarding fertilization in the Decapoda can be formulated. If we accept Binford's results then we must admit that they are contrary to everything that we know regarding fertilization in animals.

Summary.

1. The spermatozoa of the black-clawed crab, *Lophopanopeus bellus* (Stimpson) Rathbun, are minute, greenish cells, which appear like spheroids when seen from the top or bottom and like ellipsoids when seen from the side.

2. The structure of these spermatozoa is very similar to that of the edible crab, *Cancer magister* Dana. In the centre there is a tube-like central body, and surrounding this in order of sequence is a secondary vesicle, a primary vesicle and a nuclear cup with slender radiating arms.

3. There are four types of spermatozoa produced in *Lophopanopeus bellus*, depending on the number of radial arms which they contain. There are three-, four-, five- and six-rayed spermatozoa, with the four- and five-rayed types predominating in numbers.

4. In sea water and isotonic solutions of various salts, no change occurs in the normal appearance of the spermatozoa. In hypotonic solutions of these salts the spermatozoa explode by an eversion of the two vesicles and the central body.

5. In ovarian fluids some of the spermatozoa explode violently, with a rupture and disintegration of one or both vesicles.

6. Acidulated sea water has a harmful effect on the spermatozoa, either causing swelling or shrinkage, with subsequent disintegration.

7. A lowering of the osmotic pressure in the medium that

surrounds the spermatozoa, undoubtedly brings about their explosion.

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DESCRIPTION OF PLATES.

All figures in the accompanying plates were made with the aid of the camera lucida. All figures, except Figs. 3–7, were made from living spermatozoa. Figs 3–7 are drawings of spermatozoa which were fixed by osmic acid fumes and stained with Heidenhain's iron-hæmatoxylin and acid-fuchsin. The magnification of Figs. 1–7 is 3,300 times; that of Figs. 8–33 is 1,400 times, and that of Figs. 34–46. is 1,700 times.

Explanation of Plate I.

Figs. 1 and 2. Bottom and side views of living spermatozoa suspended in crab's body fluid. *b*, central body; *h*, protoplasmic cup; *v*, primary vesicle; *v′*, secondary vesicle.

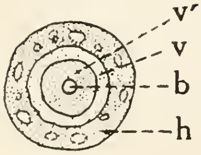
Fig. 3. Side view of spermatozoön fixed in osmic acid fumes and stained with iron-hæmatoxylin and acid-fuchsin. *b*, central body; *n*, nuclear cup; *r*, radial arms; *v*, primary vesicle; *v′*, secondary vesicle.

Figs. 4 to 7. Bottom views of spermatozoa fixed in osmic acid fumes and stained with iron-hæmatoxylin and acid-fuchsin, showing, respectively, three-, four-, five- and six-rayed types.

Figs. 8 and 9. Spermatozoa in sea water.

Figs. 10 to 19. Spermatozoa in various concentrations of sodium chloride.

Figs. 20 to 23. Spermatozoa in various concentrations of calcium chloride.



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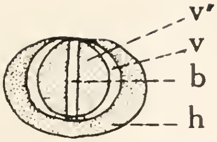

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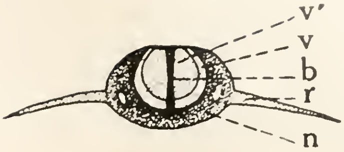

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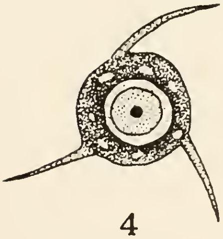

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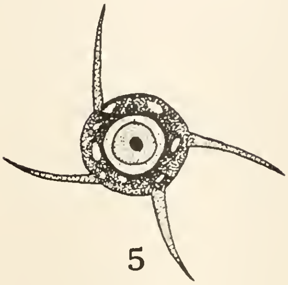

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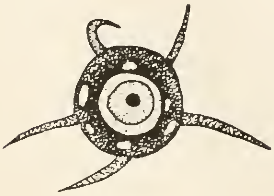

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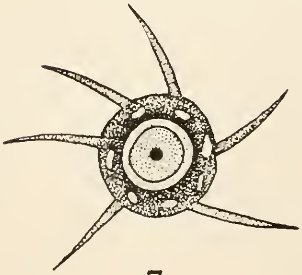

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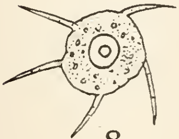

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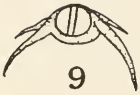

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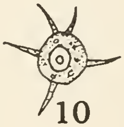

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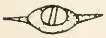

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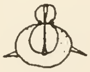

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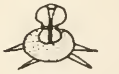

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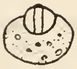

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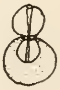

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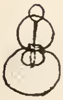

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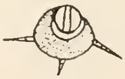

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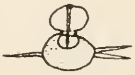

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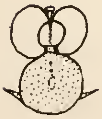

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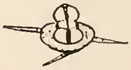

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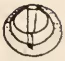

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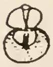

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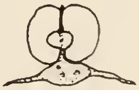

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NATHAN FASTEN.

Explanation of Plate II.

Fig. 24. Spermatozoön which has exploded in a hypotonic solution of calcium chloride.

Figs. 25 to 28. Spermatozoa which have been exposed to various concentrations of potassium chloride.

Figs. 29 to 31. Spermatozoa in weak solutions of potassium hydroxide.

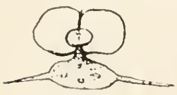
Figs. 32 and 33. Spermatozoa which have exploded in distilled water.

Figs. 34 to 39. Spermatozoa which have been subjected to the effects of ovarian fluids.

Figs. 40 to 43. Spermatozoa which have been acted on by glacial acetic acid in sea water.

Fig. 44. Bottom view of spermatozoön which has been exposed to saponin in sea water.

Figs. 45 and 46. Spermatozoa which have been acted on by nitric acid in sea water.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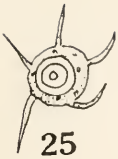
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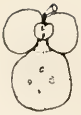
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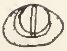
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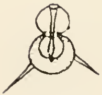
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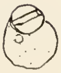
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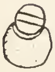
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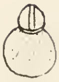
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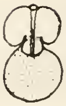
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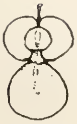
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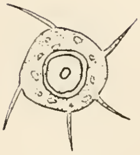
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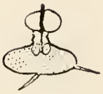
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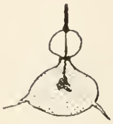
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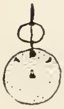
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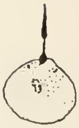
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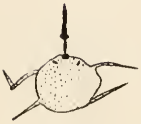
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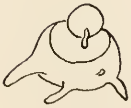
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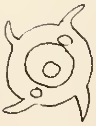
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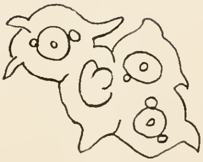
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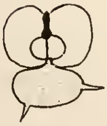
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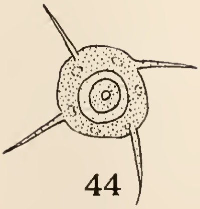
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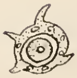
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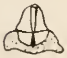
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NATHAN FASTEN.

A LEAF MIMICKING FISH.[1]

CARL H. EIGENMANN AND WILLIAM RAY ALLEN.

Heckel, in Johann Natterer's "Neue Flussfische Brasilien's nach den Beobachtungen und Mittheilungen des Entdeckers,"[2] described a small fish 3.5 inches long from a forest pool along the Rio Negro. He named it *Monocirrhus polyacanthus* and stated that at Marabitanas it was called *pirá-cáa* which means leaf-fish. Marabitanas is less than one degree north of the equator near the fiftieth degree of west latitude, in other words about sixty miles south of the southern end of the Rio Cassiquiare. No other specimens have been recorded. Günther placed it with *Polycentrus* to constitute the family Polycentridæ. The leaf-like appearance evidently impressed the Indians about Marabitanas who were acquainted with it and had a name for it.

During the Centennial Expedition of Indiana University, Dr. Allen secured three specimens of the same, or of a similar species from a brook near the Rio Itaya at Iquitos on the Peruvian Amazon.

The junior author reports that this fish was collected on September 19, 1920, while a guest of Don Antonio Layet at his hacienda, about six kilometers up the Rio Itaya from Iquitos. It was found in a small, sluggish brook which flows over very flat second bottom land, seldom inundated, and in the midst of dense forest.

There had not been much recent local rain, and there was only a slightly perceptible trickle of current at the riffles. Most of the brook was now reduced to quiet pools ten to fifteen feet across, densely overhung by vegetation, and shaded except for an hour or two at mid-day. The water was clear and of a slightly brown color, the bottom brushy, and matted with fallen leaves.

"Sr. Layet's servants had just introduced poison for me at the riffles, allowing it to flow slowly into the pools. Others with their long knives had made paths by which the low bank could be followed, The poison used was the milky sap washed from the pounded roots of *cube* (or *barbasco*), a plant cultivated as a fish poison and insecticide wash for cattle.

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[1] Contribution from the Zoölogical Laboratory of Indiana University, No. 183.

[2] Ann. Wien Mus., 1840, II., p. 439.

"I was beginning to grow impatient at the slowness of the poison, and to wonder if our long wait was going to be useless. I had observed several different species of fishes but they did not seem to be yielding to the usual respiratory difficulties following *cube*-poisoning, nor even to be trying to escape past the seines which we had stretched across the brook above and below.

"In order to know if there was sufficient current to carry the poison to every part of the pool, I began tossing broken twigs on the water to observe their course with the current. One such twig had reached a standstill, when directly beneath it I saw what was apparently a dead leaf being wafted past the twig. I couldn't understand why the twig was not moving too. At about that moment the leaf moved out into a path of sunlight, and toward the surface. There the resemblance to a fish became apparent, especially to one in search of the same. Its behavior, too, was like that of a poisoned fish struggling for oxygen."

The outline of the fish is similar to that of an asymmetrical leaf. The erected spinous dorsal and anal with their serrated character are not unlike the toothed edge of a leaf. The mimicry in color and markings is very close, the photograph and drawing of the dead specimens scarcely doing it justice. The lateral band has a position like that of a midrib of an asymmetric leaf. Like a midrib it fades away before reaching the distal margin. A petiole is not lacking, for the sharp, elongated snout and the protractile barbel carry out the resemblance.

While this fish may fall short of the perfection in mimicry exhibited by Kallima, it does take due account of the fact that few perfect leaves exsist, especially by the time they have reached the water. The transparent dorsal and soft anal between the spinous fins and caudal peduncle resemble breaks in the margin of a leaf. Futhermore the faded and discolored portions of many leaves, due to fungi, have their counterpart in the more ashy triangular area in the forward half of the fish.

The mimicry of *M. mimophyllus* has a physiological side. When swimming it moves in a gliding manner (like a seahorse) that resembles a drifting leaf. This movement is due chiefly to the rapid beating of the small transparent soft dorsal and soft anal. These fins are set within the outline of the body, their bases being transverse to the body length. They have the direct forward push of the screws of a ship. Being hyaline their motion does not attract attention.

Several other species of fishes in the forest pools have the color of dead leaves. The others were seen before yielding to the poison, while *M. mimophyllus*, with a much more complete mimicry, was not.

A technical diagnosis of the species follows.

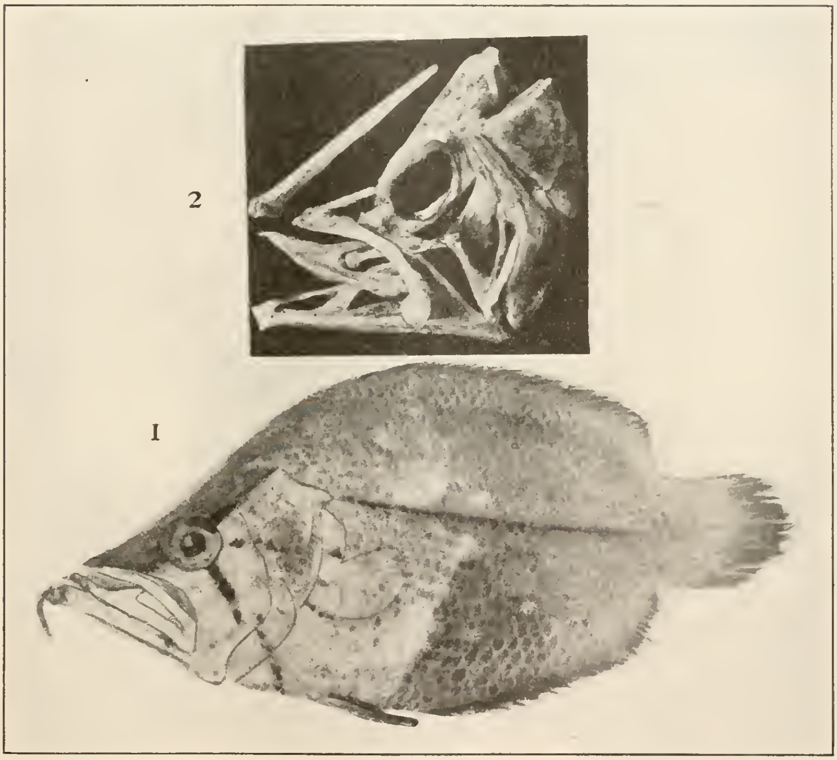

FIG. 1. Photograph of a specimen 61 mm. over all.

FIG. 2. Photograph of the skeleton of the head. The premaxillary spine is broken off from the rest of the bone, and the posterior end of the premaxillary has slipped upward a little away from its original position.

MONOCIRRHUS MIMOPHYLLUS Eigenmann & Allen spec. nov.

15715, I., 3, 44, 47, and 51 mm. long to base of caudal (65 mm. over all). Brooks near the Rio Itaya, Iquitos. Collected by Dr. W. R. Allen.

Evidently closely allied to *M. polyacanthus* Heckel, if distinct. In *M. polyacanthus* the caudal is said to be emarginate, the lateral band is said to run through the lower half of the tail, and the edge of the dorsal, anal, and tip of the ventrals are said to be blackish, the end of the caudal white.

Head 2.5; depth 1.92; D. XVI or XVII, 13; A. XII or XIII, 12 to 14.

Greatly compressed, the snout very sharp, the chin projecting, with a goatee barbel; the two rami of the mandible in contact below, equal in length to the head behind the anterior nares; maxillaries equal to snout and eye; premaxillaries greatly protractile; eye 1.5 in snout, 4 in the head, about .8 in the interorbital; opercular spine on a line between the upper margin of the orbit and the upper margin of the caudal peduncle. Profile between snout and occiput concave; gill-membranes somewhat united, entirely free from the isthmus, entirely hidden by the rami of the mandible.

Tongue very long and slender, rod-like, the free portion about as long as the eye, its tip soft, curved up and slightly cupped; premaxillary spine extending far beyond the eye, equal to the length of the mandible; mandible with one, in part two series of minute, recurved teeth; premaxillary with a single series of teeth on the sides, a triangular patch of teeth at the tip; no teeth on roof of mouth.

Pectoral broad, its length about 3 in the head, soft-rayed; distance between tip of the snout and origin of the dorsal a little more or a little less than 2 in the length without caudal; base of the spinous dorsal 2 in the length; base of soft dorsal about one-fifth of the length of the spinous dorsal; caudal *rounded*, equal to snout and eye or a little shorter; origin of anal and third dorsal spine equidistant from tip of snout; base of spinous portion of the anal about three in the length; base of soft part of anal a trifle longer than base of soft dorsal; ventrals reaching origin of anal, their inner ray adnate. First ray stout and spinous. Cheeks, opercle, and top of head to tip of snout scaled; preorbital the only portion of the head naked. Scales of sides regularly imbricate, without lateral line pores; dorsal and anal partially depressible into a scaly sheath, the spines alternating when depressed. The scales of the sides roughened on half their exposed part, margined with very fine hyaline spinules.

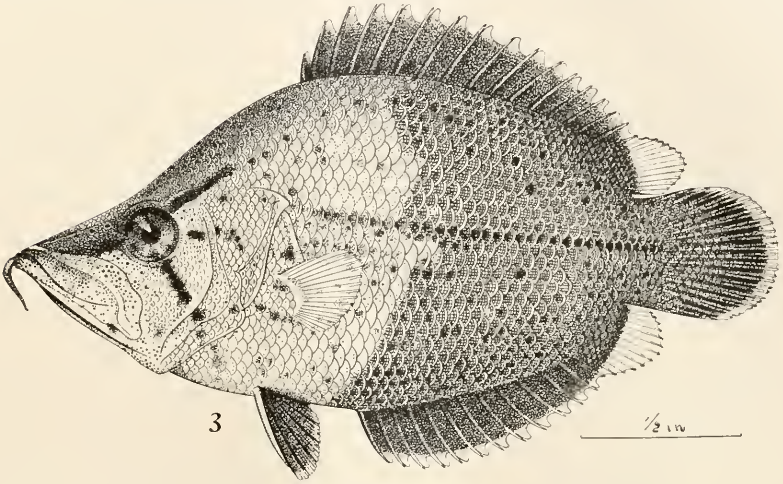

FIG. 3. Drawing of another specimen by W. S. Atkinson.

Four gill arches, lower arch of the first one with eleven rakers, the first a spinulous patch without projection, graduated to the last one which is about two thirds as long as the eye; all of them with numerous small spines; only two spinulous cushions on the upper arch; pharyngeal teeth similar to those on the gill-rakers.

Pectorals, soft dorsal, soft anal, margins of spinous dorsal, spinous anal and caudal and to a less extent of the ventrals hyaline; the hyaline of all but the soft dorsal, soft anal and pectoral bordered by black; a dark line from above the upper angle of the gill opening through the middle of the eye to the maxillary, a similar line from the eye through the cheeks crossing the breast half way between the ventrals and the gill opening, another one extending straight back from the eye; a similar dark line extending from the point of the opercle to the middle of the caudal peduncle; area from the middle of the ventrals up to the dorsal and then forward below the line through the eye to the mandible several shades lighter than the back or the area behind this line. Slightly coppery color in living fish, this shade lost in alcohol.

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THE EFFECT OF IODINE AND IODOTHYRINE ON THE LARVÆ OF SALAMANDERS. II. THE RELATION BETWEEN METAMORPHOSIS AND LIMB DEVELOPMENT IN SALAMANDER LARVÆ.

E. UHLENHUTH.

(From the Laboratories of the Rockefeller Institute for Medical Research.)

In previous experiments (1) on the larvæ of *Ambystoma opacum* I found that iodothyrine did not accelerate the development of the limbs, although it caused rapid metamorphosis. Consequently, if the administration of iodothyrine was begun at an early larval stage, the metamorphosed salamanders possessed hind limbs which did not have the full number of toes. In agreement with these observations is the fact that feeding of thymus gland, although it resulted in an inhibition of metamorphosis, did not retard the development of the limbs of the thymus-fed salamander larvæ (2). Hence it is evident that in salamander larvæ the development of the limbs is independent of the substance (thyroid hormone) which causes metamorphosis.

The relation between limb development and metamorphosis as it exists in salamander larvæ is of especial interest, since apparently it is just the opposite of what should have been expected from the experiments performed on the anuran tadpoles. Through the work of Gudernatsch (3) and many other investigators it is well known that in tadpoles administration of thyroid gland, iodothyrine and other thyroid preparations accelerates not only metamorphosis, but also the development of the limbs. Lately Swingle (4) found that the administration of inorganic iodine which causes precocious metamorphosis of the tadpoles likewise accelerates development of the limbs.

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Recent investigations have shown that the thyroid hormone and probably other morphogenic hormones, by increasing the rate of certain fundamental reactions, have the ability of causing structural changes throughout the entire organism, bringing thus about morphological expressions of a wide range affecting nearly the whole body. It seems that these hormonic substances, as far as their immediate effect is concerned, act chiefly by inducing a general histolysis throughout the various organs of the organism. There can be no doubt, however, that, besides these hormones referred to above, other substances play an important rôle in the development of an organism; these substances seem to possess a more localized action, effect the development of only certain organs and are concerned chiefly with the building up of the structures of these organs. Certainly, in the evolution of the organisms, the acquirement of the ability of elaborating the latter kind of substances must have played a rôle equally important as that played by substances such as the thyroid hormone. The limbs of the amphibians are apparently organs whose development seems to depend chiefly on the action of substances belonging to the latter group of substances and not on the activity of the thyroid hormone.

On account of the increased importance which, in the light of such considerations, seemed to attach itself to the finding that the development of the salamander limbs is independent of the thyroid hormone, it appeared necessary to repeat my previous experiments on the relation between limb development and metamorphosis. The present article will be devoted to reporting these new experiments. They consisted in causing precocious metamorphosis of the larvæ of *Ambystoma maculatum* by keeping them in iodothyrine, whereby special attention was paid to a possible acceleration of limb development. The result was the same as in the experiments on *A. opacum;* the rate of the development of the limbs remained unchanged, although metamorphosis took place at an early date. Not only larvæ, but also embryos, at early stages, were exposed to the influence of iodothyrine, in order to avoid the objection that failure of the iodothyrine to cause accelerated limb development was due to the experiments having been started at a stage at which limb development was too far advanced. Again these experiments were completely negative as to an acceleration of limb development. In order to be certain that the method employed in my experiments on salamanders was correct, several tests were carried out on tadpoles; these were positive.

In addition to these experiments, several experiments were made to test the influence of inorganic iodine on the development of the amphibian limbs. Like Swingle (4), I found a distinct acceleration of the limb development in tadpoles; in the salamander larvæ, however, iodine had no effect on the development of the limbs. The bearing of this result, which is different from that of the experiments with iodothyrine will be referred to in the discussion.

Experiments on Tadpoles.

The experiments on tadpoles, which were intended to serve as a check to the experiments on salamanders, were not advanced beyond a merely preliminary stage, since they gave positive results from the very beginning. As they are confirmative of the observations reported by other investigators, they will be reported only briefly.

In one experiment on the larvæ of *Rana sylvatica* the controls were kept under observation till the 66th day after hatching. At this time the hind limbs of the control larva furthest advanced possessed 3 distinctly differentiated toes, while the 2 other toes were still rudimentary. In the iodothyrine series (kept from the 18th to the 26th day in water which contained 0.005 gm. Bayer's iodothyrine per 1,000 c.c. of water) the fore limbs broke through the walls of the gill chamber on the 33d day after hatching in every one of the 3 larvæ surviving, at this date, from the 6 larvæ composing the series at the beginning of the experiment. The inorganic iodine, in the concentration used in this experiment (2 to 3 drops of a 1/20 M solution of iodine per 1,000 c.c. of water), as well as in other experiments, proved to be considerably less effective than the iodothyrine, as 66 days after hatching the fore limbs had not perforated the gill chamber in a single instance. Yet the limbs, the hind limbs as well as the fore limbs, were distinctly further differentiated than in the controls of the same age; the hind limbs possessed 5 fully differentiated toes and in shape were much like the hind limbs of an adult frog. Moreover, in 2 larvæ of the iodine series, which died at an age of 50 and 56 days respectively, the hind limbs possessed already at that date 5 fully differentiated toes.

In another experiment 5 series of the tadpoles of *Rana sylvatica* were kept in different concentrations of iodine (varying from 1 to 10 drops of a 1/20 M solution of iodine per 1,000 c.c. of water). The controls were kept under observation for 83 days; at the end of this period the hind limbs had remained undifferentiated, whitish buds in 5 of the 8 larvæ, while in the other 3 larvæ differentiation had taken place, the best differentiation being represented by 4 toes on the foot of the hind limbs. Many of the larvæ kept in inorganic iodine died; none of these was further advanced than the surviving larvæ. Among the surviving larvæ none had metamorphosed at the termination of the experiment, nor had the fore limbs broken through in a single instance; yet the limbs were considerably further differentiated than in the controls. In one larva of the iodine series, at an age of 73 days, the foot of the hind limbs was differentiated into 5 toes; in another larva, at an age of only 59 days, the hind limbs possessed 5 toes, and the fore limbs, which could be seen vigorously moving in the gill chamber, had developed 3 toes.

There can be no doubt that iodothyrine when administered to tadpoles greatly accelerates development of the limbs. Inorganic iodine, although it seemed less efficient in these experiments than iodothyrine, likewise increases the rate of the development of the limbs.

Experiments on Salamander Larvæ.

As pointed out in the introduction, my previous experiments on the larvæ of *Ambystoma opacum*, in which the administration of iodothyrine did not accelerate development of the limbs, were open to the criticism that the administration of iodothyrine was started at a stage at which limb development was fairly advanced (the toes having begun to differentiate), and that for this reason the iodothyrine may have been incapable of accelerating limb development. Therefore it seemed necessary to start the experiment at a very early stage. Two experiments were carried out, both on the embryos of *Ambystoma maculatum*.

Experiment I.—One egg mass of *Ambystoma maculatum* was collected on April 18, 1920. The eggs were not only freed from the general mass of jelly, but also the individual egg envelopes were removed, in order to assure ready access of the iodothyrine and iodine to the developing embryos.

Beginning of the experiment: 28 embryos selected; 9 of them placed into iodine-free water (10,000 c.c. H_2O, 0.16 gm. Na_2CO_3, 0.04 gm. K_2CO_3, 0.4 gm. $MgSO_4{-}7H_2O$, 0.6 gm. $CaCl_2$), 9 into iodine (2 drops of a 1/20 M solution of inorganic iodine per 1,000 c.c. of iodine-free water) and 10 into iodothyrine (0.01 gm. Bayer's iodothyrine per 1,000 c.c. of iodine-free water). In all embryos the first four visceral arches are formed; the fore-limb rudiments not yet differentiated from the pronephridial protuberance; no hind limbs.

The concentration of the inorganic iodine was increased to 8 drops per 1,000 c.c. 5 days, decreased to 6 drops 7 days, and decreased to 4 drops 11 days after the beginning of the experiment.

Sixteen days after the beginning of the experiment: Neither the iodine nor the iodothyrine had produced any influence on the development of the limbs (see Table I.).

Table I.

Experiment I.: 16 Days after Beginning of Experiment.

	Total Number.	Development of Toes.		
		3.5	3.0	2.5
Water	8	7		1
Iodine	8	5	1	2
Iodothyrin	10	9		1

Twenty days after the beginning of the experiment: The concentration of the iodine is decreased to 3 drops per 1,000 c.c. of water, the concentration of the iodothyrine increased to 0.1 gm. per 1,000 c.c. of water.

Twenty-seven days after the beginning of the experiment: Hind limbs commenced to differentiate into toes; but neither iodothyrine nor iodine accelerated limb development as compared to limb development of controls kept in iodine-free water (see Table II.).

TABLE II.

EXPERIMENT I.: 27 DAYS AFTER BEGINNING OF EXPERIMENT.

	Total Number.	Development of Toes.		
		6.0	4.5	4.0
Water	8	3	5	
Iodine	7		4	3
Iodothyrin	10		2	8

Yet the influence of the iodothyrine on metamorphosis had become noticeable in spite of the early stage of the larvæ, as the gills as well as the fin of the tail are found to be greatly atrophied.

Experiment II.—In this experiment one series of the embryos of *Ambystoma maculatum* was kept in inorganic iodine and one in iodine-free water. The concentration of iodine was 1 drop of a 1/20 M iodine solution per 1,000 c.c. of iodine-free water in the beginning, was increased later on to 8 drops and then gradually decreased to 3 drops. The embryos were at an early stage (formation of neural folds), when the experiment started; only the common jelly mass was removed.

TABLE III.

EXPERIMENT II.: 10 DAYS AFTER BEGINNING OF EXPERIMENT.

	Total Number.	Fore Limb Buds Present, No Indication of Toes.
Water	15	15
Iodine	14	14

TABLE IV.

EXPERIMENT II.: 18 DAYS AFTER BEGINNING OF EXPERIMENT.

	Total Number.[1]	Development of Toes.	
		3.5	2.5
Water	14	14	
Iodine	13	7	6

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[1] Several eggs and embryos were attacked by moulds and as they disintegrated or developed abnormally, they had to be discarded causing thus the decreases in the total numbers.

TABLE V.

EXPERIMENT II.: 30 DAYS AFTER BEGINNING OF EXPERIMENT.

	Total Number.[1]	Development of Toes.		
		6.0	4.5	4.0
Water................	14	5	7	2
Iodine................	12	1	10	1

As Tables III., IV. and V. show, the inorganic iodine had no influence whatsoever on the development of the limbs.

The relation between limb development and metamorphosis was further tested in two experiments in which larvæ of *Ambystoma maculatum* at a more advanced stage were employed. Concerning the action of iodothyrine, the results were in complete accordance with those obtained in the larvæ of *Ambystoma opacum;* rapid metamorphosis, but no influence on limb development was observed. In each experiment one series was devoted to the study of the influence of inorganic iodine; this substance likewise had no influence on limb development, but unlike iodothyrine it did not cause precocious metamorphosis. Both iodine experiments as regards the influence of iodine on metamorphosis were described in detail in a previous article (5); they will be only briefly reported in this article.

In Experiment III. the larvæ were placed into iodine-free water containing 0.1 gm. iodothyrine per 1,000 c.c. of water at an age of 20 days, at which date nearly all larvæ had developed 4 toes in the fore limbs and several had commenced to develop the first 2 toes in the hind limbs. Thirty-three days after hatching—*i.e.,* 13 days after the first administration of iodothyrine—every one larva metamorphosed (as compared to 101 days in the controls), but in none of them the number of toes was more than 7.5, and in one it was only 6.0, this stage of limb development corresponding to the control series kept in iodine-free water without the addition of iodothyrine.

In Experiment IV. a smaller dosis of iodothyrine (0.01 gm. per 1,000 c.c. of iodine-free water) was administered. Precocious metamorphosis was caused also by this dosis, but the development of the limbs again remained completely unaffected as compared to the controls. Since, however, metamorphosis took place at an age at which normally the limbs are fully developed (as shown by the controls), the precociously metamorphosed salamanders possessed in this experiment fully developed limbs.

Concerning the influence of inorganic iodine in these experiments, it was shown in a previous article (5) that administration of iodine does not result in precocious metamorphosis of salamander larvæ. In this article it should be added that it did have no effect also on the development of the limbs.

Discussion.

The experiments reported in this article confirm fully the observations made in my previous experiments. In the larvæ of salamanders the development of the limbs can not be accelerated by the administration of iodothyrine. Therefore, if iodothyrine is administered in doses which cause metamorphosis before the time at which, under normal conditions, the limbs are fully developed, metamorphosis takes place before the completion of limb development.

These facts demonstrate that in salamanders limb development is independent of the substance (thyroid hormone) which causes metamorphosis. This conclusion has recently been supported by several other facts. *Typhlomolge rathbuni,* the Texan cave salamander, does not possess a thyroid gland (6) and consequently does not metamorphose; yet its limbs develop in a normal manner. Hoskins and Hoskins (7) have shown that in the larvæ of *Ambystoma* the limbs develop normally, if the larvæ are deprived of their thyroid glands in early embryonic stages. This season I have repeated these experiments. Larvæ of *Ambystoma maculatum* were thyroidectomized at an early embryonic stage; these larvæ which are believed to have been successfully operated on (histological examination has not been made as yet) showed the same rate of limb development as the controls. Several larvæ of *Ambystoma tigrinum* were thyroidectomized at a stage at which 3 toes of the hind limbs were developed; the two other toes developed at a normal rate after thyroidectomy. These facts prove that in salamanders the substances causing limb development are not identical with those causing metamorphosis and consequently are not identical with the thyroid hormone.

In tadpoles substances which cause precocious metamorphosis also accelerate the development of the limbs. I have suggested, in a previous article (8), that in spite of this external difference existing between the larvæ of *Anura* and *Urodela* the primary effect of the thyroid hormone may be the same in both groups of animals, and the difference may not be a fundamentally different reaction upon the thyroid hormone, but merely a different mode of limb development.

There is no doubt that, except for the development of the limbs in tadpoles, the immediate effect of the thyroid hormone is, in both groups, predominantly a breaking down of tissues throughout the whole organism, not a building up of new organs. It is possible that in tadpoles the same substances endowed with a merely localized action cause limb development as in salamanders, but that in tadpoles these substances can not commence to build up the structures of the limbs before some obstacle has been cleared away by the action of the thyroid hormone. That the thyroid hormone controls limb development in the tadpoles does not necessarily mean that it has any part in the constructive processes of limb development. If we consider the advanced stages of the development of the fore limbs in tadpoles, we find conditions which make it indeed very probable that the thyroid hormone, in this process, plays merely the rôle of removing an obstacle external to the tissues of the limb itself. In salamanders both hind and fore limbs develop freely, while in tadpoles the fore limbs are inclosed in the gill chamber. In order that they may break through the walls of the gill chamber, certain changes of the skin and the tissues underlying it must take place; these changes are not caused by the legs themselves, but take place even in the absence of the limbs (9) at the time at which metamorphosis occurs. I have pointed out repeatedly that in salamanders one of the most conspicuous effects of the thyroid hormone is a certain change of the skin which finally results in the shedding of the skin and may be identical with the process which leads to the atrophy of the gills. A similar change is brought about in the skin of the tadpoles; in the tadpoles, too, the skin is shed for the first time when metamorphosis takes place. It is possible that the change of the skin which is necessary to permit of the freeing of the fore limbs is identical with the change that causes the first shedding of the skin.

In support of this view is the fact that the freeing of the fore limbs is clearly the only step in the development of the limbs, which in not one single instance has been observed to take place in tadpoles which had been deprived of the thyroid secretion, while the developmental processes preceding the freeing of the limb may take place in thyroidectomized tadpoles. Allen (10) states that in tadpoles of *Bufo,* in the complete absence of a thyroid, both fore and hind limbs develop normally (and even grow larger than the limbs of normal larvæ)—*i.e.,* behave exactly like the limbs of salamander larvæ—yet the fore limbs fail to break through the walls of the gill chamber. Apparently in this anuran species the development of the limbs, except for the freeing of the fore limbs, is independent of the thyroid hormone as it is in the urodelan larvæ.

Should these views prove to be correct, it would seem probable that the mode of limb development in amphibians is the morphologic expression of the existence of two kinds of morphogenic substances; one group of these substances serves to procure the actual building stones of the morphological structures of the organ, while the other group of substances merely brings about a general histolysis of old structures, removing thus obstacles to the action of the substances belonging to the first group of substances.

As has been mentioned above, the ineffectiveness of inorganic iodine in the limb development of salamanders has a reason different from that of the ineffectiveness of iodothyrine. In a previous article (5) I have shown that, in contradistinction to iodothyrine, the administration of inorganic iodine does not produce precocious metamorphosis in salamander larvæ. The inorganic iodine has no effect on the salamander metamorphosis, because the thyroid hormone in salamanders is not released during the greater part of the larval period, and a greater supply of inorganic iodine, even if it should result in the elaboration of an excess of thyroid hormone, as it actually does in tadpoles (11), can not make itself felt in the salamander larvæ in consequence of the retention of the hormone. As has been shown in this article, inorganic iodine, like iodothyrine, has no effect on limb development of salamanders. But it must be borne in mind that the ineffectiveness of inorganic iodine in limb development of salamanders is merely due to the above-mentioned peculiarity of the thyroid apparatus of the salamander larvæ and not to the fact that limb development in salamanders is independent of the thyroid hormone and of metamorphosis. Inorganic iodine could not accelerate limb development of salamanders, even if iodothyrine would have an accelerating effect.

SUMMARY.

1. Tadpoles of *Rana sylvatica* were kept in iodothyrine and in solutions of inorganic iodine. Both substances were found to accelerate limb development. This result confirms the observations of previous investigations.

2. Embryos as well as larvæ, in early stages, of *Ambystoma maculatum* were kept in iodothyrine and in inorganic iodine. Neither of these substances accelerated the development of the limbs, although iodothyrine caused rapid metamorphosis of the salamander larvæ.

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MICRODISSECTION STUDIES, III. SOME PROBLEMS IN THE MATURATION AND FERTILIZATION OF THE ECHINODERM EGG.

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This paper is a record of operative work on the starfish, sea-urchin and sand-dollar eggs to ascertain the morphological nature of changes which take place in the egg during its maturation and fertilization. Results were obtained on the effect of nuclear material on cytoplasm, the nature of cortical changes in the maturing and fertilized egg and the difference between cortex and medulla of the egg with respect to fertilizability and to other life activities. The dissection and injection of the living eggs were carried out at first by means of Barber's ('14) apparatus and later with an improved micromanipulator of my own design ('21[b]). A description of the technique as applied to microdissection has already been published (Chambers, '18[a]). A detailed description of the new micromanipulator will appear both in the *Journal of Bacteriology* and in the *Anatomical Record*.

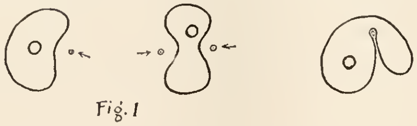
1. The Germinal Vesicle in the Maturing Starfish Egg.

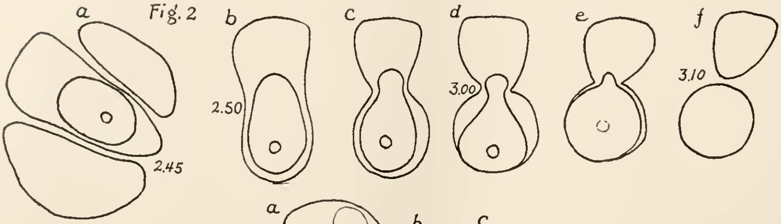
Starfish eggs, on being shed naturally, have already begun maturing. In order, however, to secure large quantities of eggs, it has been the general custom to remove the ovaries bodily from a ripe female and to cut them up in a bowl of sea water. This procedure brings the eggs into the sea water in the immature condition with germinal vesicles intact. The germinal vesicle begins to disappear anywhere from thirty to fifty minutes after the eggs come into contact with the sea water and maturation usually proceeds in a normal manner (Wilson and Mathews, '95).

The undisturbed germinal vesicle or nucleus of a fully grown immature egg is a hyaline sphere containing a sharply differentiated nucleolus and occupying about one fifth the volume of the egg. With the microdissection needle the vesicle may be moved about in the fluid cytoplasm without injury to the egg. With the needle one may considerably indent the surface of the vesicle. On removal of the needle the vesicle reverts again to the spherical shape (Fig. 1). The vesicle possesses a morphologically definite surface membrane inclosing an optically homogeneous liquid (cf. Chambers, '18[b]). Within this liquid lies a visible body, the nucleolus. By agitating the vesicle the nucleolus may be made to occupy any position within the nuclear fluid. The nuclear membrane is very easily injured. If, however, a microneedle be carefully inserted into the nucleus, the membrane about the puncture adheres to the body of the needle and the tip of the needle may push the nucleolus about with no apparent injury. The existence of considerable tension in the nuclear membrane is shown in the following experiment. An egg was cut into three fragments in such a way that the surface film forming over the cut surfaces of the middle fragment pressed upon the nucleus, deforming it considerably (Fig. 2). The attempt of the nucleus to return to a spherical shape bulged out one end of the egg fragment until it was constricted off from the remainder of the fragment (Fig. 2*b*–*f*).

Tearing the nuclear membrane in most cases results in a destruction of the nucleus. In a few cases it was possible to produce a slight rupture with no noticeable injurious effects. Such a case is recorded in Fig. 3. At 10:44 A.M. undue pressure on the germinal vesicle when cutting an immature egg in two resulted in its rupture followed by a lobular extrusion bounded by a very delicate film. During the following ten minutes the vesicle began slowly to revert to its original shape (Fig. 3*b* and *c*). Before that was attained the maturation process began and, at 10:55, the outline of the vesicle had disappeared. The nucleated egg fragment maturated normally and five hours and a half after insemination it had segmented in two. At 8:40 P.M. it had developed into a swimming blastula.

The cytoplasm of the egg allows of considerable tearing without apparent injury (Chambers, '17-*a*). If, however, the nuclear membrane be torn, a very striking phenomenon occurs. The cytoplasm immediately surrounding the nucleus disintegrates and liquefies. If the rupture of the nucleus be violent, the disintegration of the cytoplasm spreads rapidly until the entire egg is involved. If the rupture be slight, the disintegrative process is quickly limited by a surface film which forms on the boundary between the disintegrating and the surrounding healthy cytoplasm (Fig. 4). This film tends to prevent any further spread of the destructive process. The destruction of the cytoplasm is evidently due to something which emanates from the injured nucleus. The injury to the cytoplasm does not start where the nuclear membrane is first torn, but from the entire surface of the injured nucleus.





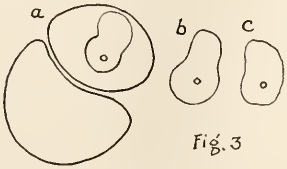


FIG. 1. Figures showing the extent to which the nucleus (germinal vesicle) of an immature starfish egg may be indented on one or both sides without rupture. On removing the needle the nucleus reverts to its original spherical shape.

FIG. 2. *a*, immature starfish egg cut at 2:45 P.M. into three parts; the nucleus has remained intact but is laterally compressed in the middle fragment. *b, c, d, e* and *f*, successive steps in attempt of nucleus to round up; *b*, 2:50 P.M.; *d*, 3:00 P.M.; *f*, 3:10 P.M.

FIG. 3. *a*, partial rupture of nucleus followed by a repair of its membrane. *b* and *c*, successive changes in the shape of the nucleus within the following ten minutes after which time it disappeared.

This is analogous to results obtained by injuring red blood corpuscles with a needle upon which hemoglobin escapes immediately from the entire surface (Chambers, '15).

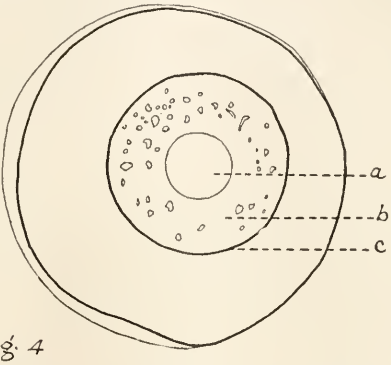


FIG. 4. Disintegration of cytoplasm surrounding the nucleus on tearing the nucleus with a needle. (*a*) Faint hyaline sphere, a remnant of the destroyed nucleus. (*b*) Disintegrated cytoplasm. (*c*) Cytoplasmic surface film separating disintegrated from healthy cytoplasm.

Within the nucleus itself the immediate effect of the injury is a dissolution of the nucleolus. A nuclear remnant tends to persist after the injury as a hyaline sphere lying within the disintegration products of the cytoplasm. On being touched with the needle it fades from view.

In permanently immature eggs, such as eggs which have been standing in sea water for an hour or more without maturing, the disintegrative effect on the cytoplasm by injuring the nucleus tends to be much more restricted, and the nuclear sphere which persists after the injury can be shown to possess a morphologically definite membrane. Such a sphere is easily dissected out of the egg. Frequently, when the germinal vesicle lies close to the periphery of the egg, the disintegration of the cytoplasm quickly reaches the surface. With the formation of a surface film over the healthy cytoplasm the disintegrative area lies in a deep bay on one side of the egg. This hollow is slowly obliterated as the semi-fluid substance of the egg strives to assume a spherical shape. In this way the disintegrated material is forced out of the egg together with the persisting nuclear sphere. This nuclear sphere persists for some time in the sea water. It can be deformed by means of the needle and, on tearing its surface, the fluid contents escape, leaving behind a collapsed membrane which disappears within 10 to 15 seconds.

Fig. 5 shows the effect of cutting the mature egg nucleus of the starfish egg. By pushing the nucleus against the inner surface of the egg it is possible to pinch it into two pieces. Each piece rounds up but, if the two are allowed to come into contact, they will fuse into a single nucleus again. The same result obtains in the sand-dollar and sea-urchin eggs. If, however, the nuclear membrane be torn, a disintegration of the cytoplasm results analogous to that produced on rupturing the germinal vesicle. The extent of disintegration is much more limited, owing doubtless to the much smaller amount of nuclear material present. Similar results were obtained on tearing the nucleus of the *Arbacia* egg.

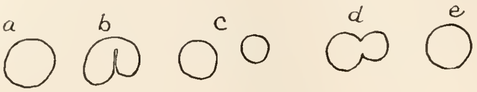
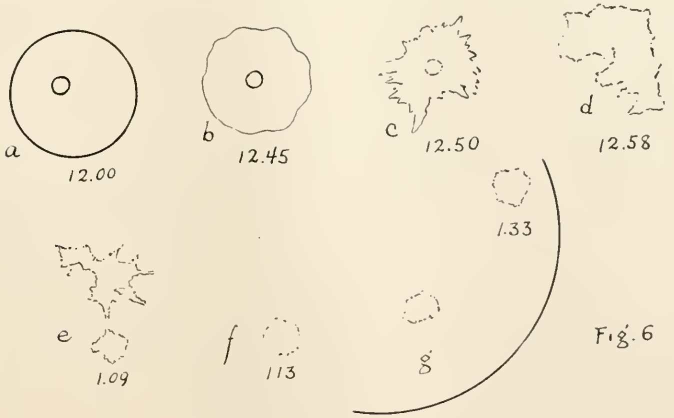


Fig. 5

Fig. 5. Effect of cutting mature nucleus of a starfish (*Asterias*) or sea-urchin (*Arbacia*) egg. *a*, intact egg nucleus; *b*, nucleus in process of being cut in two. The nucleus was pushed against the periphery of the egg as it was being cut by a vertical needle; *c*, the separated fragments of the nucleus; *d*, reunion of the fragments; *e*, reconstituted nucleus.

It was found possible to destroy the cytoplasm of one egg by injecting into it nuclear material obtained from another egg. This experiment has to be performed very rapidly, for if the nuclear material be allowed to remain longer than ten seconds within the pipette it has no effect whatever when injected into the cytoplasm of an egg. If it be injected within that time the destructive effect is very pronounced.

If an egg be allowed to undergo normal maturation, the germinal vesicle disappears except for a small remnant which becomes the definite egg nucleus. This egg nucleus moves to the surface of the egg, where it gives off the two polar bodies. It then constitutes the female pronucleus, which remains quiescent until fertilization occurs. The disappearance of the germinal vesicle is a well-known phenomenon. In order, however, to locate definite stages selected for my operations I introduce the following summary. The germinal vesicle with an intact membrane is shown in Fig. 6. Within thirty to forty-five minutes after standing in sea water the nuclear membrane exhibits wrinkles and its outline begins to fade from view. Within a few minutes no membrane is visible and cytoplasmic granules can be seen moving into the region hitherto occupied by the nucleus, while the nuclear sap appears to be diffusing out (Fig. 6-*c*). As the nuclear membrane disappears the nucleolus fades from view. The invasion of the nuclear area by cytoplasmic granules continues until all of the area except a small portion is rendered indistinguishable from the general cytoplasm of the egg. This small portion persists as the egg nucleus (Fig 6*e* and *f*). In Fig. 6-*g* two consecutive positions of the nucleus are shown. At 1 : 13 P.M. it lay deep in the substance of the egg. In twenty minutes it had moved to the periphery of the egg preparatory to the formation of the polar bodies.



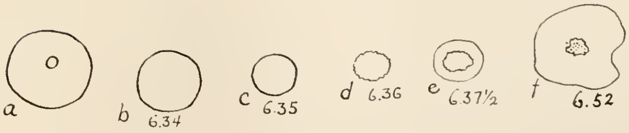


Fig. 7

FIG. 6. Camera lucida drawings of the successive steps in the normal dissolution of the germinal vesicle in the maturing starfish egg. The process was somewhat slowed down owing possibly to the compressed condition of the egg necessary for detailed observation.

FIG. 7. *a*, intact germinal vesicle within the egg. *b*, nucleus after having been torn out of the egg and brought into sea water. *c*, *d*, *e* and *f*, successive changes undergone by the nucleus lying in sea water.

By means of the microdissection needle it is possible to show, at the stage shown in Fig. 6-*d*, that the membrane of the germinal vesicle no longer exists. By careful manipulation it was possible to push the cytoplasmic granules into the nuclear area. A slight rapid movement of the needle, however, was sufficient to give rise to disintegrative processes similar to those on tearing an intact germinal vesicle. In the normal maturation process the mingling of the nuclear sap with the cytoplasm is very gradual, being completed in the case recorded not under ten minutes. It is this gradual mixing which apparently prevents disintegration.

Morgan ('93) and Mathews (Wilson and Mathews, '95) found that maturation was accelerated by shaking starfish eggs shortly after they were placed in sea water. They concluded that the shaking ruptured the membrane of the germinal vesicle and so allowed the nuclear material to mix more quickly with the cytoplasm. I have repeatedly tried to intermix cytoplasm and nuclear material by rupturing the nuclear membrane of the starfish egg with the needle, but in every case I get an explosive disintegration of the cytoplasm. The ruptured nuclear membrane which Mathews (W. and M., '95) and Marcus ('07) describe in fixed and stained immature eggs which had been violently shaken is possibly the membrane of the sphere which I found to persist after injury to the germinal vesicle (see page 321). It is more likely that the shaking which accelerates processes within the egg leads to the normal gradual dissolution of the nuclear membrane and the subsequent diffusion of the nuclear material throughout the egg. I have been able to do this occasionally with the needle. An intact germinal vesicle which to all appearances should take fifteen to twenty minutes to go into dissolution will often immediately exhibit a wrinkled outline on being gently agitated with the needle. Then follows the gradual fading from view of its outline with the subsequent changes as shown in Fig. 6.

The intact germinal vesicle may be brought into the sea water by tearing away the surrounding cytoplasm. During the process the nucleolus fades from view. The slightest tearing of the nuclear surface then causes the entire liquid vesicle to disappear in the water. If, however, the nucleus be left alone, it shrinks for a time and then swells. The changes appreciable to the eye are shown in Fig. 7. During the swelling of the nucleus a substance apparently separates out which collects into a small mass and persists as a gelatinous body. It is possible that this abnormal separating out is analogous to the formation of the definitive egg nucleus in the normal process of maturation. This separating out of a gelatinous material from a liquid nucleus upon injury may be similar to the method of precociously inducing chromosomes in spermatocytes of the grasshopper (Chambers, '14).

2. The Existence of an Extraneous Membrane About the Unfertilized Egg.

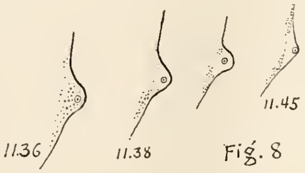
The existence of a membrane about the unfertilized egg rising off as the fertilization membrane upon insemination was first suggested by the earlier investigators (*e.g.,* Hertwig, '76; Herbst, '93). Kite ('12) and Glaser ('13) agreed with them whereas McClendon ('14), Harvey ('14) and Elder ('13) claimed that the fertilization membrane is a new formation consequent to fertilization. Heilbrunn ('13) also identifies it with the actual protoplasmic surface of the egg, which he considers to be in a state of a gel and which lifts off as the fertilization membrane, a new surface film forming over the egg underneath it.

My experiments indicate that the unfertilized eggs of the starfish, sea-urchin and sand-dollar all possess a membrane extraneous to their true protoplasmic surface, and that it is this membrane which, upon insemination, is lifted off as the well-known fertilization membrane.

In the unfertilized egg the membrane is more or less tightly glued to the surface of the egg just as Kite ('12) described it. In the sea-urchin egg it is extremely delicate and can be demonstrated only as follows (Fig. 8): The needle is inserted as nearly as possible through the periphery of the egg and left there. Within a few seconds the protoplasm, lying immediately under the egg membrane and distal to the needle, flow away from the needle until the needle lies in a small protuberance which is formed by a very slightly lifted portion of the egg membrane.

The existence of the egg membrane is easily demonstrated in the starfish egg. In Fig. 9 the disintegration of the cytoplasm following injury to the germinal vesicle has reached the surface of the egg. The disintegrated area is quickly localized by a surface film bounding a cup-shaped depression on the surface of the egg. Roofing over the depression is the egg membrane. The egg membrane can also be shown by cutting an egg in two by pressing the egg against the coverslip with the side of a needle. The pressure of the needle cuts the egg in two without rupturing the membrane, which, on releasing the egg, bridges the gap between the pieces and holds them together (cf. Figs. 11 and 12, page 329).

The difference between the consistency of the egg membrane in the starfish and the sea-urchin egg is strikingly shown in the following experiments. With the eggs in a hanging drop the egg is pressed against the coverslip with the side of a glass needle until



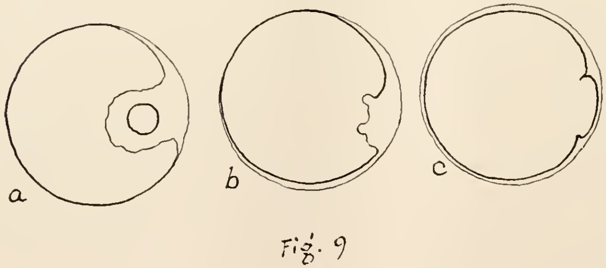


FIG. 8. Needle inserted at 11:36 A.M. through periphery of a sea-urchin egg and left there. At 11:38 the cytoplasmic granules have been flowing away from the needle. A new surface film begins to appear with the needle left outside. At 11:45 the original egg membrane appears as a delicate membrane partially lifted off the surface of the egg by the needle.

FIG. 9. Lifting of a membrane from the surface of an immature starfish egg following injury to the egg. *a*, local disintegration of cytoplasm following destruction of the germinal vesicle (cf. Fig. 4). An egg membrane becomes apparent as the cytoplasm retreats from it. *b* and *c*, gradual separation of the membrane all over the surface of the egg.

the pressure divides the egg into two pieces. In the sea-urchin egg the two pieces immediately round up and roll away from one another. In the starfish egg the tougher membrane is not ruptured, but holds the two pieces together.

The membrane of the sea-urchin egg is so delicate that it is also possible to cut the egg in two in the following manner: In a hanging drop the horizontal end of the needle is brought *over* the egg (Fig. 10). The needle is now lowered. This brings the needle against the upper surface of the egg and presses the egg down against the surface film of the hanging drop. On lowering the needle still further it passes through the egg and out of the drop, cutting the egg cleanly in two. In the case of the starfish egg this procedure would drag the egg out of the drop along with the needle. The membrane of the sand-dollar egg is weaker than that of the starfish and stronger than that of the sea-urchin egg.

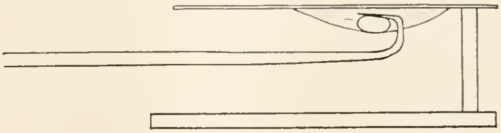


Fig. 10

Fig. 10. Side view of moist chamber to show one method of cutting an egg in two with the microdissection needle.

The consistency of the membrane varies with the age of the egg. The full-grown immature egg of the starfish has a relatively tough membrane. On the other hand, young ovarian eggs possess very delicate membranes and they can be cut in two with the same ease as mature sea-urchin eggs.

The strongest argument regarding the existence of a membrane about the unfertilized egg is that a membrane may be stripped off the egg whereupon the egg, which was previously non-adherent, now sticks to everything it touches. The fertilizability of such naked eggs is discussed under the next heading.

The existence of egg membranes is a fairly universal feature and it is, therefore, not surprising that we should find them in the echinoderm eggs which have generally been considered as naked. The unfertilized *Cumingia* egg has an extremely tough membrane, so tough that it is difficult to rupture it without completely destroying the egg contents. The vitelline membranes in the frog and in the chick are undoubtedly analogous structures.

3. The Egg Membrane and the Fertilization Membrane are Identical.

Prior to fertilization no membrane enveloping the egg is visible. Upon fertilization a membrane lifts off which can easily be cut away from the egg. Figs. 11 and 12 indicate the identity of a preexisting membrane with the fertilization membrane. Fig. 11-*a* shows an egg cut in two with an investing membrane holding the pieces together. Upon fertilization the membrane lifts off, enclosing the two pieces in a single cavity (Fig. 11-*b*). One only of the pieces happened to segment, and the fact that the two pieces lie in one cavity is shown in Fig. 11-*c*, where the blastomeres of the segmented portion have encroached on the area around the nonsegmented piece. In Fig. 12 an egg was cut into three pieces, the egg nucleus lying in one of the pieces. Upon fertilization the membrane lifted off the pieces, each of which received sperm and developed into swimming larvæ. Fig. 12-*c* shows the empty fertilization membrane after the three larvæ had escaped. In Fig. 13 is shown an egg which, on being cut in two, was rolled about in an attempt to separate the pieces. The egg membrane between the two pieces was twisted into a thread joining the two. Upon fertilization each piece exhibited a complete fertilization membrane, but the fact that the two investing membranes are portions of one common membrane is shown by the connecting thread.

A conclusive test for the starfish and sand-dollar egg is the removal of the egg membrane prior to insemination. Occasionally, pricking the egg is sufficient to elevate the membrane. No subsequent development takes place. It is possible, however, to remove this membrane by tearing it and the egg then be made to slip out. This is more easily done on eggs which have been standing for some time in seawater. On catching at the surface of such eggs with the needle, the membrane is often torn in such way that the egg slips out leaving the membrane stuck to the needle. Such an egg, when inseminated, is fertilized and subsequently segments with no investing membrane whatever.

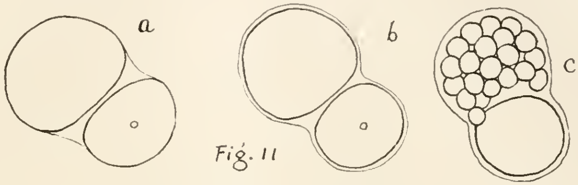


Fig. 11

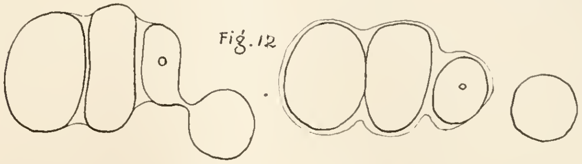


Fig. 12

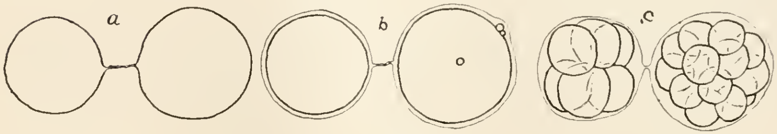


Fig. 13

FIG. 11. *a*, starfish egg cut in two without destroying the investing membrane. *b*, after insemination the investing membrane lifts off both fragments as the fertilization membrane. *c*, one of the fragments segmented, the other did not. That both fragments lie in a common cavity is shown by the encroaching of blastomeres of one fragment into the region of the unsegmented fragment.

FIG. 12. *a*, starfish egg cut into three pieces. One piece was squashed and produced an exovate. *b*, on being fertilized the exovate was pinched off as an endoplasmic sphere (cf. Fig. 25). The rest of the fragments produced a common fertilization membrane. Each of the three enclosed fragments developed into a swimming larva.

FIG. 13. *a*, sand-dollar egg rolled as it was cut in two. The egg membrane between the two pieces was twisted into a thread joining the two. *b*, egg shortly after fertilization showing fertilization membrane about each connected by a filament. *c*, the two pieces in an early segmentation stage.

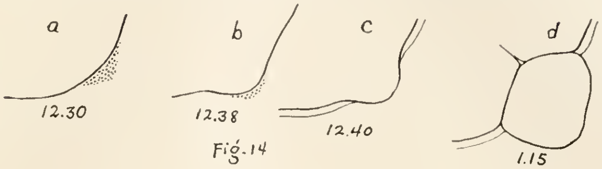
The difference in reaction of sperm to an egg which has been denuded of its membrane as well as of its jelly, and to one which has not is very striking. An egg within its membrane is quickly surrounded by spermatozoa as they are trapped in the jelly surrounding the membrane. In a membraneless egg no crowding of spermatozoa is noticeable and heavy insemination is necessary to bring about fertilization. When a cloud of sperm has been blown upon a naked egg, one may frequently observe a spermatozoön swim toward it, wander over its surface, and then swim away. On the other hand, the empty membrane with its investing jelly immediately becomes covered with a halo of spermatozoa. This observation accords with the interpretation of Buller ('02), that the investing jelly determines the direction of the sperm which are captured by it, and that there is no apparent chemotactic substance excreted by the egg to attract the sperm.

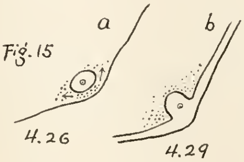
The difference in position of the polar bodies in the starfish egg with respect to the fertilization membrane as shown by Gemmill ('12) (see also Chambers and Mossop, '18, and Garrey, '19) may be explained as follows: When the polar bodies form prior to fertilization they rise off the surface of the egg, carrying with them the closely adherent membrane. When they are pinched off the egg membrane remains continuous about the egg and subsequent insemination results in the formation of a fertilization membrane with the polar bodies lying outside. If, however, the eggs are inseminated before extrusion of the polar bodies, the egg membrane lifts off as the fertilization membrane and, when the polar bodies are formed, they lie within the membrane.

In the sea-urchin egg the identity of the egg membrane with the fertilization membrane is more difficult to demonstrate. In Fig. 14 is shown the effect of locally injuring the surface of the sea-urchin egg. In *a* is a disintegrated mass produced by tearing a spot on the surface with a needle. In *b* this area is shown as a bulge which may be explained as being produced by the interior pressure of the egg on a surface weakened by the loss of an investing membrane. In *c* the egg has been fertilized. The fertilization membrane is formed over all the surface except at the injured place. In *d* segmentation has occurred and a blastomere protrudes through the gap in the fertilization membrane.

A better demonstration is the case shown in Fig. 15. At 4:26 the tip of a needle was punched through the cortex. Within a few seconds the cytoplasm distal to the needle flowed away, leaving the needle lying under a delicate membrane (Fig. 15-*a*). At 4:27 the egg was inseminated with the needle still in place. At 4:29

the fertilization membrane was formed, showing its continuity with the delicate membrane previously noticeable (Fig. 15-*b*).





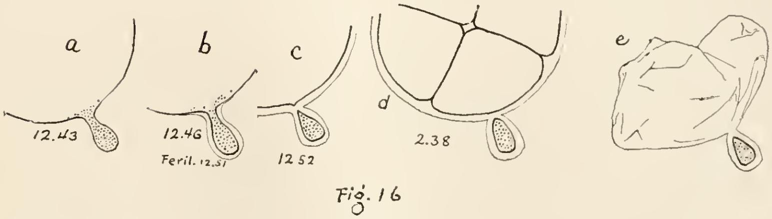


FIG. 14. Sea-urchin egg with surface torn producing local cytolysis. *a*, a new surface film has formed under the cytolyzed area which is being extruded. *b*, a bulge appears in the region of the new surface showing this region to be weaker than elsewhere on the egg surface. *c*, egg after fertilization exhibiting a fertilization membrane over the egg except at the place previously torn. *d*, the same egg 35 minutes later with a blastomere protruding through the tear.

FIG. 15. *a*, needle piercing sea-urchin egg near its periphery. The cytoplasmic granules are flowing in the direction of the arrows. One minute later the egg was inseminated. *c*, an intact fertilization membrane forms, inclosing both egg and needle tip.

FIG. 16. *a*, protrusion on surface of egg produced by pulling at cortex with needle. *b*, three minutes later the investing membrane lifted off surface of protrusion. *c*, one minute after fertilization. The protrusion has been pinched off from the egg and its investing membrane can be seen to be continuous with the fertilization membrane. *d*, empty and collapsed fertilization membrane.

In the sea-urchin egg the membrane often rises off a protrusion caused by pulling at the cortex with the needle. Such a case is shown in Fig. 16. The protrusion was formed at 12:43. At 12:46 a membrane had lifted off the protrusion. At 12:51 the egg was inseminated, and one minute later the membrane was found continuous with the fertilization membrane. The protrusion subsequently pinched itself off and persisted in a sac-like protuberance of the fertilization membrane (Fig. 16-*d*–*e*).

In all of the various eggs studied a change in the consistency of the membrane takes place very soon after it has been elevated. The membrane, at first very soft and delicate, progressively toughens until it becomes almost parchment-like during the later segmentation stages. It is of interest to note that Harvey ('10) found a difference between the unfertilized and the fertilized sea-urchin egg when subjected to sulfuric acid. The acid dissolves the unfertilized egg completely, whereas it dissolves all of the fertilized egg except the fertilization membrane. Some chemical change apparently takes place as the membrane lifts off the egg.

Outside the membrane is a considerable zone of a structureless jelly. In the sand-dollar egg the jelly very loosely adheres to the membrane. On cutting into the jelly the egg with its membrane easily slips out. This is to a somewhat lesser degree true for the starfish egg. In the starfish egg one often sees the under surface of the jelly pushed away from the surface of the unfertilized egg by the protruding polar body.

The question as to whether the membrane lifts off the surface of the egg or whether the egg shrinks leaving the membrane behind has been raised by Glaser ('14) in spite of McClendon's ('10) statement to the contrary. Glaser, by making a large series of measurements, claims that the egg shrinks upon fertilization, and that the initial diameter of the completed fertilization membrane is equal to that of the unfertilized egg. Glaser's measurements were made on the assumption that the eggs always maintain a spherical shape. This is not true. The mature unfertilized egg is very soft and if allowed to lie on the bottom of a glass dish tends to flatten into the shape of a disc. Upon fertilization the egg rounds up as the fertilization membrane leaves its surface. One can readily see if the observations are taken of eggs in one plane only that erroneous conclusions may be arrived at.

I used two methods to ascertain the diameter of starfish eggs before and after fertilization. One method was to place a drop containing a few eggs on a gelatin-coated slide. The eggs were rolled over by means of a micro-needle and only those which maintained their spherical shape were measured. With a micro-pipette sperm were introduced into the drop without disturbing the relative positions of the eggs. A second method was to place several eggs in a hanging drop in a Barber moist chamber. By piercing the surrounding jelly with a needle the egg to be measured could be held suspended in the middle of the drop. Numerous measurements of the starfish egg were made at different times through several summers and in every case the egg maintained its original size as the fertilization membrane rose off its surface. Not only does the egg not decrease in volume, but it slightly *increases* in size until segmentation occurs. The accompanying table is one sample of the measurements made:

	Un-fertil.	Minutes after Fertilization.					
		1″	2″	7″	10″	20″	70″
Egg diameter	3.4	3.4	3.4	3.4	3.5 x 3.55	3.5 x 3.6	3.5 x 3.6
Fertilization membrane diameter		3.5	3.6	3.65 x 3.7	3.65 x 3.7	3.75 x 3.75	3.9 x 3.9

The conclusions from this table apply both to starfish and sea-urchin eggs. They may not necessarily be true for other species.

Fig. 17 shows successive steps in pulling a starfish egg out of its fertilization membrane. No second membrane is ever formed even with superimposed insemination. Occasionally the hyaline plasma layer in such an extruded egg swells up and simulates a second membrane, and it is probably this that has been described by certain investigators as a second fertilization membrane. The hyaline plasma layer will be discussed under heading 5.

An unfertilized mature sea-urchin egg may be rolled about and its contents churned to the extent of producing "fountain currents" within the egg (Chambers, '17-*b*). This is done by pushing an egg in a drop shallow enough to compress the egg. Currents are produced which flow backward immediately under the surface of the egg and forward along its central axis (Fig. 18). By careful manipulation it is possible to do this without rupturing the investing membrane. Such an egg is capable of forming a normal fertilization membrane when inseminated. If the pushing process be carried too far, a distinctive quiver can be recognized, as of something giving way. On subsequent insemination such eggs produce a collapsed fertilization membrane. The quiver undoubtedly was due to a rupture of the egg membrane. On account of this rupture the fluid, which presumably collects under the membrane, leaks out and the membrane is not lifted uniformly.

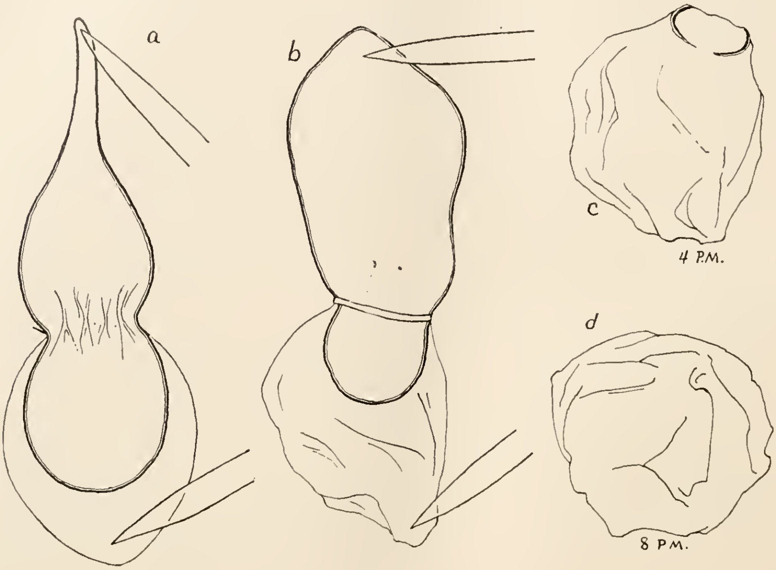


Fig. 17

FIG. 17. *a* and *b*, successive steps in pulling a starfish egg out of its fertilization membrane. *c*, empty membrane at 4:00 P.M. *d*, ditto four hours later at 8:00 P.M. The membrane persists as a collapsed remnant for a long time.

4. The Cortex and Interior of the Unfertilized Egg.

The cytoplasm of the immature starfish egg is uniformly semi-solid. A gash made in it with a needle is maintained for some minutes before closing up. When the germinal vesicle breaks down naturally, the egg protoplasm becomes more fluid so that a gash through such an egg quickly closes up. The cortex—*i.e.*, the surface of the egg immediately beneath the egg membrane—tends always to remain more solid (Chambers, '17-*a*). Because of this difference in consistency the cortex and medulla of the egg can be separated from one another as follows ('21^{a}): If the surface of the mature starfish egg be torn with a needle and the egg then be caught at the opposite side and pulled to the edge of the hanging drop, the compression on the egg produced by the shallow water at the edge of the drop will cause the fluid interior to ooze out through the tear to form a spherical exovate (see Fig. 25, page 344). One may so manipulate the process as to cause the egg nucleus either to remain behind in the cortex (the cortical remnant) or to pass into the extruded sphere of endoplasmic material.

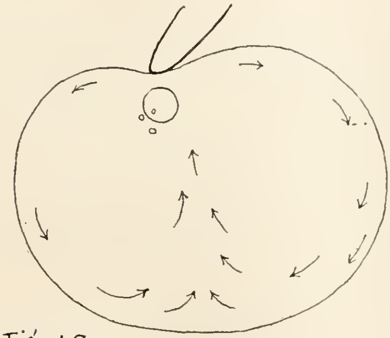


Fig. 18

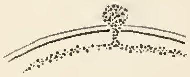


Fig. 19

FIG. 18. Currents produced within a sea-urchin egg by pushing a sea-urchin egg held against a coverslip by a shallow film of water. The direction of the currents is shown by the arrows. The nucleus, after being carried about with the current, tends to come to rest in the location shown in the figure.

FIG. 19. Part of the cortex of a fertilized egg after the appearance of the hyaline plasma layer. The cortex was ruptured in one place and cytoplasmic granules can be seen issuing through the rupture in the hyaline plasma layer and the investing fertilization membrane.

The cortical remnant is relatively solid and remains more or less inclosed within the egg membrane and its jelly. If left long enough it will eventually round up so as to present the appearance of a diminutive egg surrounded by a collapsed and wrinkled egg membrane.

The endoplasmic material which has escaped from the egg into the sea water is fluid and tends immediately to round up. On tearing with a needle its surface behaves like that of a highly viscous oil drop, adheres tenaciously to glass. As long as it possesses an intact surface it looks exactly like an egg fragment and will undergo disintegrative changes similar to those of entire eggs on being torn with the needle (cf. Chambers, '17-*a*).

The ability to produce endoplasmic spheres is possibly due to the relatively tough egg membrane in the starfish egg which helps to keep back the adherent cortex. In the sea-urchin egg, with an extremely delicate egg membrane, it has been impossible to cause the interior to flow out, as the cortex tends to flow with it.

The sand-dollar egg behaves very much like the starfish egg. The egg membrane is appreciable in the unfertilized egg and endoplasmic spheres are readily produced.

A difference in the functional activities of the cortex and interior of the starfish egg is discussed under the headings 6 and 7.

5. The Hyaline Plasma Layer.

Prior to fertilization the cytoplasmic granules in the sea-urchin and sand-dollar egg lie close to the surface. Within ten minutes after fertilization the granules have undergone a centripetal migration, leaving an appreciable peripheral zone of a hyaline appearance which has been called the hyaline plasma layer (Loeb's gelatinous film, '13, p. 19).

The microdissection needle indicates that this layer is relatively firm and gelatinous. The very fluid internal cytoplasm may be made to flow out through a rupture in this layer if the egg be torn. This is shown in Fig. 19. The cytoplasmic granules lie against the inner boundary of this layer and may be seen oozing out through the small tear in this layer and through a tear in the fertilization membrane to the exterior.

The hyaline plasma layer adheres very tenaciously to the needle and when an egg has been deprived of its fertilization membrane the egg sticks to everything it touches.

Loeb has called attention to the fact that the hyaline plasma layer in a segmented egg bridges the segmentation furrow. When the furrow is first formed, however, the hyaline plasma layer does not bridge the furrow, but is carried in on the walls of the cleavage furrow (Fig. 20-*a, b, c*). The layer is thicker in the floor of the furrow, but it is only later when the furrow has cut through the egg that the hyaline plasma layers on the opposite surfaces of the furrow run together. Each half of the segmenting egg tends to assume the shape of a sphere owing to the separation of the two asters of the amphiaster (Chambers, '17-*b*, '19). If there were no other forces at play, the two blastomeres, when formed, should be spheres. In the sea-urchin egg the adhesiveness of the hyaline plasma layer tends to draw the two blastomeres together; also the fertilization membrane, not rising to any great extent off the surface of the egg, must exert some pressure on the two blastomeres. In the sand-dollar the fertilization membrane is well

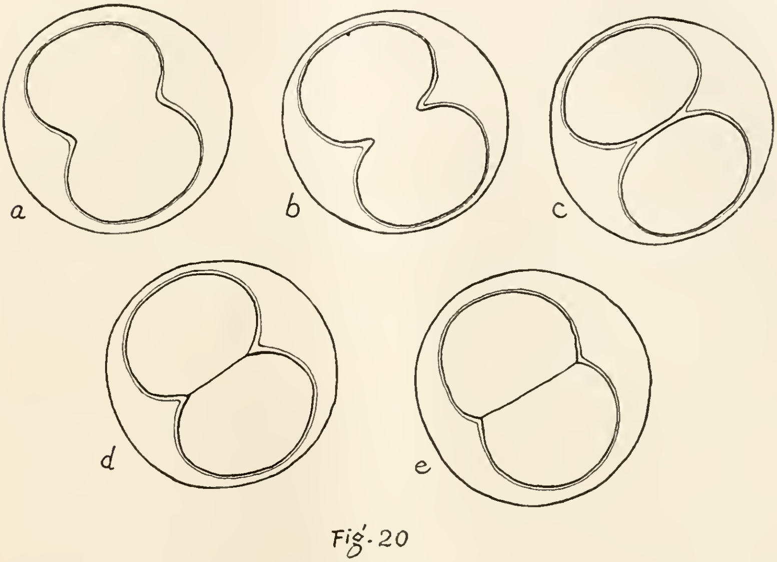


FIG. 20. Contour of a sand-dollar egg at various stages of its cleavage into two blastomeres. In *a* and *b* the hyaline plasma layer is seen carried in on the walls of the deepening furrow. In *c* the egg has segmented in two with the hyaline plasma layer on opposite sides of the furrow tending to merge into each other. In *d* this process is carried further. In *e* the two blastomeres are tending to assume the shape of hemispheres with the hyaline plasma layer bridging the furrow.

lifted, so that there is plenty of room within the membrane, permitting the two blastomeres to assume almost spherical shapes (Fig. 20-*c*). When the cleavage furrow is completed the two blastomeres are contiguous only where the two spheres touch. At this place the hyaline plasma layers of the two blastomeres merge. We have here, apparently, two opposing forces; first, the jellied aster holding each blastomere to a spherical shape, and, second, the affinity of the plasma layer substance surrounding the two blastomeres. As soon as the asters disappear and the cytoplasm of the blastomeres reverts to a more fluid state the plasma layers of the two blastomeres merge more and more and the blastomeres are pulled together till they assume shapes approaching those of hemispheres (Fig. 20-*e*). The outlines in Fig. 20 are camera lucida drawings taken during the successive stages of one sand-dollar egg.[1]

In the starfish, where there is no appreciable hyaline layer, and where the fertilization membrane is lifted far beyond the surface

[1] It has recently been intimated that the microdissection method is unreliable as a means of ascertaining changes in viscosity in the dividing egg because of supposed discrepancies in the results obtained by Seifriz ('20) and myself ('17^b and '19). As a matter of fact the results of Seifriz harmonize perfectly with mine. Seifriz states "there is a pronounced decrease in viscosity of the central region of the cell with the first appearance of the amphiasters." This statement has been interpreted as running counter to mine. This is not true for although my results indicate that the astral portion of the amphiaster is jellied, I definitely state (p. 494, '17) that the central region and the zone between the two halves of the egg are fluid where "a distinct flow of granules medianward can be observed."

Again, on completion of cleavage Seifriz notes that the two blastomeres become liquid. This statement also fits in with my results. I state (p. 51, '19) that, immediately after cleavage and while the two blastomeres *are still spherical*, the firmness of the cytoplasm persists. Later, when the asters disappear the cytoplasm liquefies and the two blastomeres crowd up against one another. Seifriz noted this last liquid state of the two blastomeres without considering the state prior to it.

I may mention here a possible criticism of the centrifuge method in ascertaining viscosity variations. There are critical stages in the developing asters during which agitation causes their disappearance. This was noted long ago by Wilson. On bringing the eggs to rest the asters reappear and development proceeds normally. I have already discussed this matter fully ('19). The centrifuge and miscrodissection methods of studying the physical state of protoplasm should serve as valuable checks on one another, if only the investigators in these fields would agree on cooperation.

of the egg, the blastomeres are practically non-adhesive, and they maintain more or less spherical shapes till well on into the later segmentation stages.

6. The Localization of a Material Which Affects the Life of the Unfertilized Starfish Egg.

It is well known that immature starfish eggs can be kept in sea water at room temperature for 36 hours or more without disintegrating. That the germinal vesicle or nucleus is responsible for this length of life can be demonstrated by cutting an immature egg in two. The nucleated fragment lasts fully as long as the entire egg. The non-nucleated portion, on the other hand, disintegrates within three to four hours. In mature unfertilized eggs the conditions are quite different. In the mature egg the germinal vesicle has broken down and the nuclear sap has diffused throughout the egg. Loeb ('02) and Mathews ('07) showed that such eggs have a higher rate of oxidation than immature eggs and if left unfertilized disintegrate within 8 to 10 hours whereas the immature eggs last for days.

The non-nucleated fragment of the mature egg lasts as long as the whole egg, evidently owing to the dispersed nuclear sap of the dissolved germinal vesicle. What is significant is that the nucleated fragment lives no longer than the non-nucleated fragment. Both contain the dispersed nuclear sap, while the nucleated fragment possesses also the definitive mature egg nucleus which is ultimately to become the female pronucleus. Apparently it is the dispersed nuclear sap and not the definitive mature egg nucleus which is chiefly concerned. In the formation of the nucleus of the mature egg we have possibly something analogous to the state of affairs in many Protozoa where the nuclear apparatus consists of a tropho- or macro-nucleus concerned chiefly in the metabolic activities of the cell, and the kineto- or micro-nucleus which has only to do with the reproductive activities. In the starfish egg we may consider the germinal vesicle as a combined tropho- and kineto-nucleus. On the approach of maturation the tropho-nuclear material (nuclear sap) diffuses throughout the egg, leaving behind the kineto-nuclear part, the mature egg nucleus, which gives off the polar bodies to become ultimately the female pronucleus.

The fluid interior of the mature unfertilized egg, if isolated by being made to escape through a tear or the cortex, withstands disintegration for 24 to 36 hours. The presence of even a small part of the original cortex in organic continuity with it causes it to disintegrate in about the same time as an entire mature egg. This would indicate that the reactions which make for disintegration reside chiefly in the cortex. This, together with the fact that the cortex of the egg is necessary for fertilization, would indicate that the cortex is the seat of the initial activation processes of the egg. The relatively inactive central material of the starfish and sand-dollar egg somewhat resembles that of the *Linerges,* the Scyphomedusan, which Conklin ('08) has described. Conklin speaks of "the large cavity in the line of the first cleavage furrow filled with gelatinous or fluid substance, which forms the ground substance of the central area of the unsegmented egg." He found that most of the ground substance escapes into the cleavage cavity and suggested that it is the fluid yoke which is gradually used up in the nourishment of the embryo. The central substance of the *Linerges* egg is probably not strictly analogous with that of the starfish or sand-dollar egg. In *Linerges* cleavage is of a type peculiar to yolk-laden eggs and the central substance escapes during the first cleavage. On the other hand, in the echinoderm egg the nucleus lies well within the central substance of the egg and, upon fertilization, all of the endoplasm is used up in the formation of the cleavage asters and nothing apparently escapes into the early cleavage cavity. We can not, therefore, conclude that the interior of the Echinoderm egg consists of entirely inert material. It lacks certain essential features, but when coexistent with the cortex it plays a full part in the cleavage of the egg.

7. The Localization of a Substance Which Renders a Starfish Egg Fertilizable.

Wilson ('03^{ab}) in *Cerebratulus* and *Renilla* and Yatsu ('04 and '08) in *Cerebratulus* have shown that non-nucleated fragments of the egg are capable of fertilization only after the germinal vesicle has broken down. With more delicate methods rendered possible by the microdissection instrument it has been possible to work out this problem in detail and to ascertain to some extent the distribution of the material which renders fertilization possible.

A number of fully grown immature starfish eggs were enucleated by carefully dissecting out their germinal vesicles. None became fertilized when inseminated. In another lot of immature eggs the germinal vesicle was torn while in the egg (Fig. 21). Immediate dissolution of the nuclear membrane took place with a disintegration of the cytoplasm around the nuclear area. Those eggs which succeeded in forming a protective surface film to prevent spread of the disintegration process subsequently rounded up. Upon insemination none of the eggs showed any sign of being fertilized.

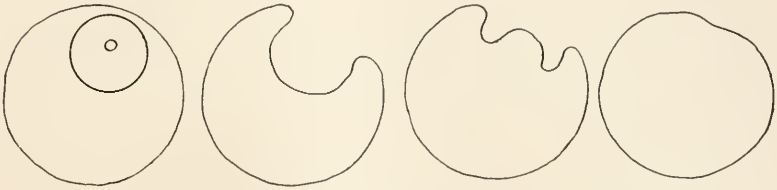
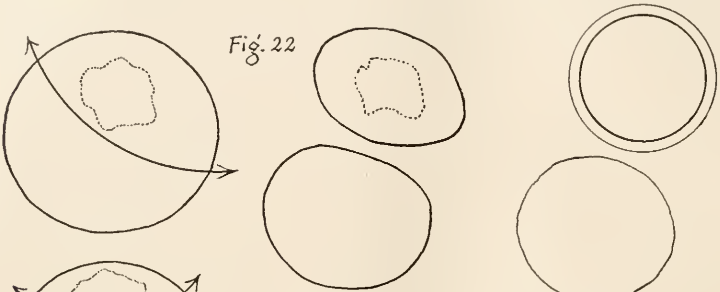


FIG. 21. A starfish egg whose germinal vesicle is eliminated by puncturing it (cf. Fig. 9). The cytoplasm surrounding this nucleus was also destroyed. This enucleated remnant is nonfertilizable.

Eggs were then taken with the germinal vesicle in various stages of normal dissolution and cut into nucleated and non-nucleated portions. The eggs may be grouped into stages *b*, *c* and *d*, according to the stage of dissolution of their germinal vesicles, as shown in Fig. 6 (page 323). Whenever the cut passed through the nuclear area during the nuclear stages *b*, *c* and *d*, disintegration always took place, involving all of the nucleated portion and a small part of the non-nucleated piece (Fig. 23 *a*, *b* and *c*). When the cut did not pass through the nuclear area all persisting nucleated portions matured normally and upon insemination formed fertilization membranes and segmented. Of the non-nucleated portions those from eggs in stage *b* are non-fertilizable (Fig. 22). Those from eggs in stage *c* form fertilization membranes upon insemination. Nuclear division also takes place, so that the egg fragment becomes multi-nucleated but remains unsegmented (Fig. 23-*c*). Non-nucleated fragments of eggs in a later stage (stage *d*) proceed somewhat farther (Fig. 24). The multi-nucleated masses arising from them make several periodic attempts at segmentation (Fig. 24-*c*). Small furrows appear over the surface of the egg, cutting in between the peripherally arranged nuclei.

Fig. 22



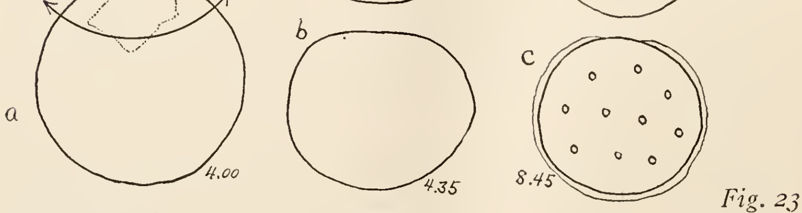


Fig. 23

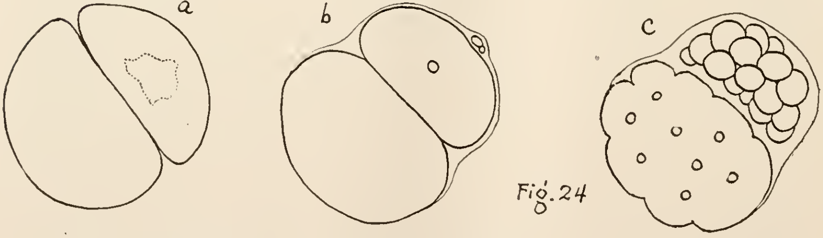


Fig. 24

Fig. 22. Starfish egg in stage corresponding to *b* in Fig. 6 cut into two fragments. The non-nucleated fragment contains no material from the germinal vesicle and is nonfertilizable.

Fig. 23. Starfish egg in a later stage corresponding to *c* in Fig. 6 cut through the nuclear area. The cytoplasm in the injured nuclear area disintegrated leaving a non-nucleated fragment, *b*. That the fragment is fertilizable is shown in *c* by the formation of a fertilization membrane and the repeated division of the sperm nucleus. The fragment, however, is unable to segment.

Fig. 24. *a*, starfish egg in stage *d* of Fig. 7 cut into a nucleated and non-nucleated fragment. *b*, both fragments fertilized. The nucleated fragment segmented in the normal way with a number of blastomeres. The non-nucleated fragment became multinucleated and furrows appeared over its surface in an attempt at segmentation.

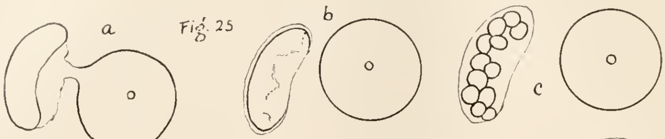
These furrows then disappear, to reappear again after a short interval. This may occur several times until the egg finally reverts to a spherical shape and remains so. In. stage *f* the germinal vesicle has disappeared except for the definitive egg nucleus. Of such eggs any non-nucleated portion down to a certain size is capable of being fertilized and undergoing cleavage.

The above experiments lead one to infer the existence of a substance in the germinal vesicle which, on dissolution of the nuclear membrane, diffuses throughout the cytoplasm. The fertilizability of any egg fragment apparently depends upon the extent of diffusion of this substance. An egg fragment taken when a minimum amount of this substance has diffused into it will allow the sperm nucleus which has entered into it to divide. The presence of a little more of this substance will allow the fragment to undergo abortive segmentation. It is not until a sufficient amount is distributed throughout the egg that any fragment can develop properly.

Mature eggs were now studied, and it was found that any egg fragment in order to be capable of fertilization must contain a portion of the original cortex. The cortex and interior of mature unfertilized eggs were separated according to the method described under heading 4 (Fig. 25 *a* and *b*). The endoplasmic sphere and the cortical remnant were then inseminated. The fragment consisting of the cortical remnant is readily fertilizable and undergoes segmentation (Fig. 25 *b* and *c*). The endoplasmic sphere is nonfertilizable, no matter whether it contains the egg nucleus or not.

That the protoplasm of the endoplasmic spheres has not been irreparably injured in the process of flowing through a small tear in the cortex is shown in the following experiment. Eggs were squashed until the endoplasm protruded as lobate processes, whereupon the pressure on the eggs was lifted and the extrusion allowed to flow back into the egg. Such eggs are fertilizable and are capable of undergoing cleavage. One such case is illustrated in Fig. 26 where the cortex was torn in two places on squashing the egg and two exovates were formed. The nucleated exovate was allowed to pinch itself off. The other exovate flowed back into the remainder of the egg upon insemination (Fig. 26 *b* and *c*). A fairly complete fertilization membrane formed around the egg except at the two torn spots and cleavage followed.

Endoplasmic exovates were also produced which remain connected by a bridge of protoplasm to the collapsed cortical portion of the egg. On being inseminated the exovate either is drawn back into the cortical portion as the latter rounds up with the formation of a fertilization membrane or is pinched off, after which it remains as an inert body.



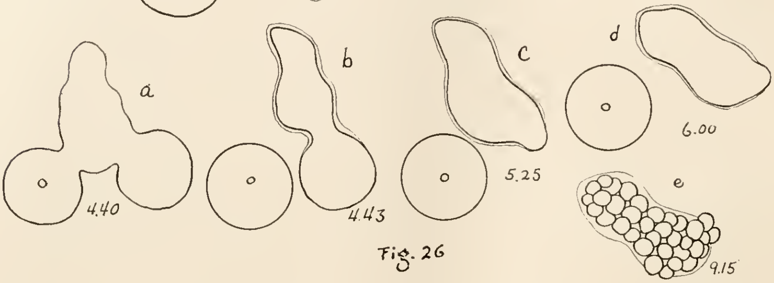


FIG. 25. *a*, nucleated exovate of internal cytoplasm produced by squashing a starfish egg. *b*, fragments inseminated after the endoplasmic sphere was pinched off. Only the ectoplasmic remnant forms a fertilization membrane. *c*, the endoplasmic sphere remains inert and nonfertilizable (cf. Fig. 12).

FIG. 26. *a*, starfish egg squashed producing two endoplasmic exovates. *b*, the nucleated exovate was pinched off. Upon insemination the other exovate drew back into the ectoplasmic remnant which formed a fertilization membrane. *c*, *d* and *e*, the ectoplasmic remnant underwent segmentation showing that the disturbance due to the squashing does not prevent segmentation. The endoplasmic sphere remains inert (*d*).

The possibility suggested itself that the substance which renders an egg fertilizable has a tendency to collect in the surface film of an egg and that, if an exovate remained in organic continuity with the egg, this substance might spread to the surface film of the exovate, thus rendering it fertilizable. Endoplasmic exovates were, therefore, produced which remained connected for varying lengths of time with the cortical portion of the egg. Some of the exovates remained connected for as long as fifteen minutes. Before insemination they were pinched off from the cortical portion of the eggs. None developed of those which were separated in such a way that there was no question as to their lacking any of the original cortex of the egg.

An endoplasmic sphere, in order to develop at all, apparently must incorporate in its substance at least a part of the original cortex of the egg. This is shown in Fig. 27. An exovate was

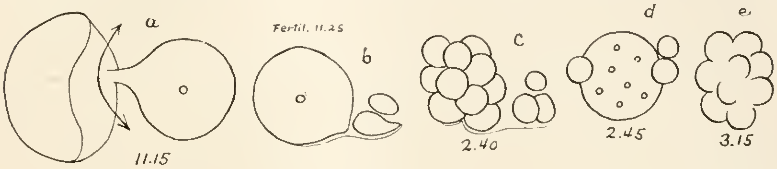


Fig 27

FIG. 27. *a*, an exovate is produced by squashing and most of the ectoplasmic part is cut away along line of arrow. *b*, the endoplasmic sphere formed itself incorporating a small part of the cortex. Upon fertilization the small cortical region formed a partial fertilization membrane. *c*, many furrows form simultaneously over the surface of the egg showing that it has been fertilized. (Note that the small cortical piece to one side of the egg has segmented in two.) *d*, the egg has reverted into a multinucleated nonsegmented mass except for three blastomere-like bodies which were pinched off. *e*, the fragment is again attempting to segment.

produced by crushing an egg (Fig. 27-*a*). However, before the exovate was set free most of the cortical remnant was cut away, leaving a very small piece which was drawn into the circumference of the endoplasmic sphere. On being inseminated a small shred of the egg membrane lifted off from this remnant, and this was all that constituted the fertilization membrane (Fig. 27-*b*). A sperm on entering this sphere underwent nuclear division several times. This was followed by cleavage furrows which formed on the surface of the egg between the peripheral nuclei and gave to the egg the appearance of a mulberry (Fig. 27-*c*). Some of the furrows deepened sufficiently to pinch off nucleated bodies. A few minutes later the furrows became obliterated and the main body of the egg appeared again as a non-segmented but multinucleated mass (Fig. 27-*d*). This process may occur several times (Fig. 27-*e*). The ability of an exovate to approximate normal segmentation is a function of the amount of the original egg cortex which it incorporates.

The inability of the endoplasmic sphere to develop is not due to the lack of successful sperm entry. Sections show that the sperm enter with ease but they remain unchanged and no asters form about them. In this regard the sperm react exactly as they do when they have entered immature eggs.

There must be something localized in the cortex which is necessary for successful fertilization and development (cf. Lillie, '14, '18). On the evidence presented here we may assume that this substance, originally within the germinal vesicle, diffuses out upon its dissolution and accumulates in the cortex of the egg. It is held in the cortex of the egg and is not carried out in the endoplasmic spheres on crushing the egg. The spheres are, therefore, incapable of being fertilized. Finally, the variation in the ability to segment among exovates containing varying amounts of cortical material indicates that there must also be a definite minimum amount of this substance present in order that an egg fragment may develop.

Conclusions.

1. The nucleus possesses a morphologically definite membrane.

2. Tearing the nucleus results in an immediate change of the nuclear membrane, followed by a disintegration of the cytoplasm surrounding it. This is most striking in the relatively large nucleus (germinal vesicle) of the starfish egg.

3. Injection of the germinal vesicle sap of one egg into the cytoplasm of another egg starts up disintegration processes in the injected area.

4. The mature egg nucleus can be pinched into two fragments. The fragments behave like fluid droplets and will run together when contiguous. Eggs whose nuclei have been operated upon in this manner are capable of normal segmentation.

5. A membrane can be demonstrated adhering to the surface of the unfertilized starfish, sea-urchin and sand-dollar eggs. This egg membrane is most pronounced in the starfish and least of all in the sea-urchin. In the starfish and sand-dollar the membrane can be stripped off without injuring the egg. In the starfish a very delicate egg membrane can be demonstrated investing half-sized

immature eggs. This membrane becomes more pronounced as the eggs reach their full growth and still more so as the egg matures. In the sea-urchin the immature eggs exhibit no trace of a membrane until the eggs begin maturation. In the mature unfertilized sea-urchin egg the membrane has reached a development comparable to that of the half-grown immature egg of the starfish.

6. The egg membrane rises off the surface of the egg upon fertilization and constitutes the fertilization membrane. No appreciable diminution in volume of the egg occurs during this process.

7. An egg, whose membrane has been removed, is fertilizable and segments without a fertilization membrane.

8. The hyaline plasma layer, which forms on the surface of the sea-urchin and sand-dollar egg within ten minutes after fertilization, binds the blastomeres together. In the starfish egg no such layer is formed, and, if the fertilization membrane be removed, the blastomeres tend to fall apart.

9. The fertilizability and approach to normal development of an egg fragment is directly proportional to the amount of a substance which emanates from the germinal vesicle during maturation.

10. The unfertilized mature egg possesses a more solid cortex of appreciable thickness inclosing a highly fluid interior. The fluid interior of the starfish and sand-dollar eggs can be made to ooze out through a tear in the cortex, whereupon it forms a surface film on coming into contact with sea water. In this way the internal and cortical material of the egg can be isolated from one another. Both round up, the internal material immediately and the cortical after some time.

11. Endoplasmic material, possessing a small part of the original cortex, is fertilizable and the approach to normal development is in direct proportion to the amount of cortical material present. The presence of even a small amount of cortical material causes disintegrative changes to set in at about the same time as in a whole egg.

12. The following table gives, for the various kinds of fragments of immature and mature starfish eggs, the length of time that they withstand disintegration when left standing in seawater and also whether they are or are not capable of being fertilized:

	Immature		Mature			
	Nucl. fragm. or entire egg	Non-nucl. fragm.	Nucl. fragm. or entire egg	Non-nucl. fragm.	Nucl. or Non-nucl.	
					Ectoplasmic remnant	Endoplasmic sphere
Longevity in hours...	24–36	2–3	8–10	8–10	8–10	24–36
Fertilizability...	+ (when mature)	−	+	+	+	−

As regards longevity it will be seen that the immature egg depends upon its nucleus (germinal vesicle) to prevent disintegration, for a fragment lacking the nucleus disintegrates very quickly. On the other hand, the mature egg, which has become permeated with the nuclear sap of the germinal vesicle, behaves quite differently. The non-nucleated fragment of a mature egg lasts longer than that of an immature egg and it is significant that the presence of the nucleus of the mature egg, which consists of not much more than the chromosomal constituents, has no effect in preventing disintegration.

The long period that the endoplasmic sphere withstands disintegration indicates that the factors which make for disintegration reside chiefly in the original cortex of the mature egg.

In regard to fertilizability it is evident that the substance which renders cytoplasm fertilizable emanates from the germinal vesicle and finally becomes localized in the cortex of the mature egg.

We can, therefore, distinguish three factors in the starfish egg; one affecting longevity, the second affecting disintegration and the third affecting fertilizability. The first and third have been traced to the germinal vesicle of the immature egg. The second is a function of the egg cortex.

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NOTE CONCERNING THE ORIGIN OF POLARITY IN THE FROG'S EGG. A CORRECTION.

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In connection with work published in 1919 (BIOL. BULL., Vol. 37: 312–361) on the modification and control of development in the frog egg, it seemed desirable to determine if possible the origin of the polarity of the egg. The position taken was that polarity must be either a matter of inheritance or of determination by factors external to the egg. If the former possibility is true the problem is, of course, simply made more remote. The second possibility, since it is known or believed that polarity arises in a number of plant and animal eggs, in response to external factors, seemed the logical one to test, especially since it is the one most readily investigated experimentally. The first question was to determine the relation, if any, between the polarity of the egg and its mode of attachment to the ovarian membrane. Here it was found and it has since been confirmed, that in 75–80 *per cent.* of the cases, the pedicle which attaches the follicle to the ovarian membrane, is located on or within 20° of the equatorial region of the egg. Since a band 40° wide over the equatorial region of a sphere involves only about 34 *per cent.* of the total area it would seem that the pedicle is not located at random over the surface of the egg but with reference to some other factor, or factors.

Since the polar axes of the ovarian eggs bear every relation to gravity this factor is made highly improbable as having any influence on the origin of polarity.

The next question to arise was the relation of the polarity of the egg to its food and oxygen supply—the blood flowing through the follicular vessels. From observations made at that time on both injected and living specimens I believed and stated, p. 321, of the above mentioned paper, that " . . . in every case observed, the greater part of the arterial blood supply was restricted to the pigment hemisphere " and that the blood supply of the unpigmented hemisphere was largely venous. It was further suggested that "the data indicate that polarity in the egg arises . . . in response to external conditions, viz., to the blood supply of the egg: that region of the oögonium chancing to be most richly supplied with arterial blood being destined to become, by virtue of this respiratory and nutritive relation, the animal pole of the egg."

It may be stated here that the problem was by no means considered solved and in 1919 plans for its further and more complete investigation were fairly well worked out. The investigation has continued with numerous interruptions and is still incomplete, but pending its outcome it has seemed desirable to make this statement.

It now appears that the previous observations were not sufficiently extensive to warrant the general statements indicated above. Certainly there is a considerable range of variation from what I thought was the typical situation and illustrated in Fig. 3 of the 1919 paper. And, it may be added, the figure is correct. But, on the other hand, cases have been observed more recently where the vegetative hemisphere was largely supplied by arterial blood, as well as various intermediate conditions. Furthermore one occasionally finds in the vessels that run to the follicle in the *mature* or nearly mature egg, a direct shunt between the small artery and vein. As far as the existence of any definite relation between the polarity of the *mature* or *nearly mature* egg and arterial or venous blood supply is concerned, I am obliged to withdraw the suggestion as it first appeared. It seems evident enough that polarity must be established early in the history of the egg, possibly in relation to the vascular supply. Supposing, as a working hypothesis, that such is the case it is conceivable that the vascularization in the follicle may change considerably especially as the egg approaches maturity—the only stage previously examined. It is along these lines that the investigation is being continued with the hope of throwing further light on the question.

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